ETHANOL PRODUCTION FROM SYNGAS BY *CLOSTRIDIUM* STRAIN P11 USING CORN STEEP LIQUOR AS A NUTRIENT REPLACEMENT

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TABLE OF CONTENTS

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

Figure Page

NOMENCLATURE

Abbreviations

[CO]	Carbonyl molecule
ACS	Acetyl-CoA Synthase
ADH	Alcohol Dehydrogenase
ADP	Adenosine diphosphate
AFEX	Ammonia Fiber Explosion
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
CoA	Coenzyme-A
CODH	Carbon monoxide dehydrogenase
CSL	Corn Steep Liquor
CSTR	Continuous Stirred Tank Reactor
EDTA	Ethylene Diamine Tetraacetic Acid
ETP	Electron Transport Phosphorylation
FIBEX	Fiber Extrusion Explosion

FID	Flame Ionization Detector
GC	Gas Chromatography
GHG	Green House Gas
GLM	General Linear Models
H_4folate	Tetra hydrofolate
HFR	Hollow Fiber Reactor
HPLC	High Performance Liquid Chromatography
LCA	Life Cycle Assessment
LPG	Liquefied Petroleum Gas
MES	2-(N-morpholino)ethanesulfonic acid
MESNA	MercaptoEthane Sulfonate Sodium
MJ	Mega Joule
MTBE	Methyl Tertiary Butyl Ether
NAD	Nicotinamide Adenine Dinucleotide
NADH	Reduced form of Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NO_x	Nitrous Oxides
OD	Optical Density
PBCR	Packed-Bubble Column Reactor

PPM	Parts Per Million
PTFE	Polytetrafluoroethylene
RPM	Rotations Per Minute
SAS	Statistical Analysis Software
SCCM	Standard Cubic Centimeters per Minute (sccm)
SHE	Standard Hydrogen Electrode
SHF	Separate Hydrolysis and Fermentation
SSCF	Simultaneous Saccharification and Co-Fermentation
SSF	Simultaneous Saccharification and Fermentation
TBR	Trickle Bed Reactor
TC	Total Carbon
TCD	Thermal Conductivity Detector
THF	Tetrahydrofolate
TN	Total Nitrogen
UV-Vis	Ultraviolet-Visible
v/v	Volume Per Volume
YE	Yeast Extract

Symbols

t	Time	h
X	Biomass Concentration	g/L
q_p	Substrate Uptake Rate	mole gas/g cells.hr
a	Gas/Liquid Interface Area Per Liquid Volume	m^2/m^3
K_La	Overall Mass Transfer Coefficient	s^{-1}
K_L	Mass Transfer Coefficient	m/s
P/V	Power Per Volume	W/m^3

Greek symbols

μ	Specific growth rate	h^{-1}
μ_{max}	Maximum specific growth rate	h^{-1}
α	Constant	
β	Constant	

CHAPTER I

INTRODUCTION

The transportation sector on a global scale is mostly dependent on fossil fuels, especially petroleum derived fuels such as gasoline, diesel, liquefied petroleum gas (LPG) and compressed natural gas (Demirbas et al. 2004). Increased fossil fuel prices, global warming due to greenhouse gas (GHG) emissions and steps to decrease the dependence on foreign oil motivated researchers to investigate new processes to produce energy efficient, non-polluting and renewable fuel such as ethanol. The first consideration in the development of ethanol was to produce it from renewable resources, followed by the determination of the cost-effectiveness and technical feasibility of using alcohol blended gasoline as a fuel in transportation sector (Goldstein 1981).

Production of fuel ethanol from domestic resources can enhance the agricultural sector, create jobs in rural areas, decrease oil imports thereby reducing trade deficits, and reduce GHG buildup (Demirbas 2005). Unlike fossil fuels, combustion of ethanol produced through fermentation does not emit particulates or NO_x because it is an oxygenated fuel, containing up to 35% O_2 by weight (Demirbas 2005; Lang et al. 2001). Carbon dioxide released by the combustion of ethanol is reabsorbed by the plants

through the process of photosynthesis to maintain carbon-neutral in the atmosphere (Goldemberg et al. 2008).

Currently, ethanol is used both as a fuel oxygenate additive and as E85 fuel (85% ethanol and 15% gasoline). In order to reduce hazardous gas emissions, the 1990 Clean Air Act mandated addition of 2% O_2 by weight to be included in gasoline. This can be achieved by blending gasoline with oxygenated additives such as ethanol and methyl-tertiary-butyl-ether (MTBE). The latter compound was identified as a predominant groundwater contaminant (Schmidt et al. 2004). Further, being a potent health hazard, MTBE use in several states such as California, Colorado, New Jersey, and Ohio has been banned (Otero et al. 2007). These reasons along with increased ethanol production have led to a decrease in MTBE production and consumption as shown in Figure 1.1 (Luchansky and Monks 2009).

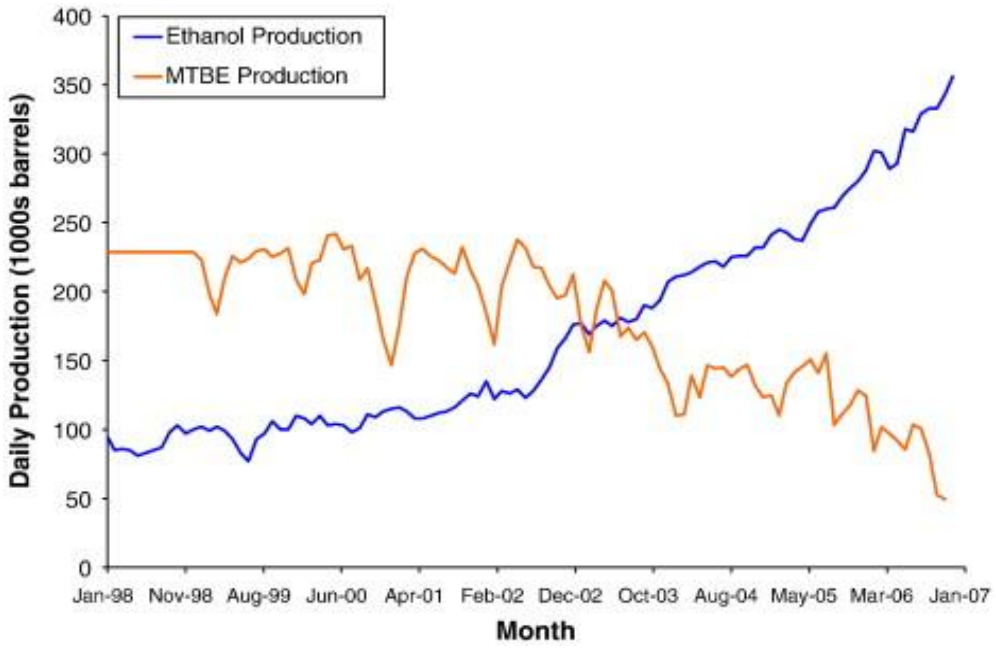

Figure 1.1 Replacement of MTBE with ethanol as gasoline oxygenate (Luchansky and Monks 2009).

Life Cycle Assessment (LCA) is considered one of the best methodologies used by the scientific community to evaluate the net environmental benefits or losses associated with biofuel production and use (Cherubini et al. 2009). Most LCA results have confirmed that there is a considerable decrease in the GHG emissions with the use of biofuels such as ethanol and biodiesel in the transportation sector (Blottnitz and Curran 2007; Kim and Dale 2005; Punter et al. 2004).

Global ethanol production in 2007 was 62.3 billion litres (Pilgrim 2009). Brazil and U.S. accounted for approximately 72% of world ethanol production (Kim and Dale 2004). Feedstocks used for ethanol production varies. In the U.S., corn is the major feedstock while sugarcane is the primary feedstock in Brazil (Wyman 1996). Presently, there are 170 biorefineries operational in the U.S. and another 20 are under construction (Renewable Fuels Association 2009). It is expected that ethanol production in the U.S. will exceed 42 billion litres by the year 2011 (Baker and Zahniser 2007).

Biofuels can be classified as first- and second-generation based on the feedstock used. First generation biofuels feedstocks such as sugars, starches and vegetable oils compete with the global food supply and often require fertile land, extensive irrigation requirements, and limited geographical availability. Second generation biofuel feedstocks such as agricultural and forest residues, wood, paper, and perennial grasses are advantageous in terms of land usage, fertility and environmental performance. The fuel produced from these feedstocks vary based on the conversion processes used such as thermochemical, flash pyrolysis, and enzymatic and chemical catalysis (Cherubini et al. 2009).

Broadly, ethanol can be produced by three major routes:

1. Direct fermentation of sugars from sugar crops.
2. Fermentation of sugars extracted by acid/enzymatic hydrolysis of starchy and lignocellulosic feedstocks.
3. Fermentation of synthesis gas generated by the gasification of lignocellulosic feedstocks.

Lignocellulosic biomass is mainly composed of cellulose (40-60%), hemicellulose (20-40%) and lignin (10-25%) (Hamelinck et al. 2005). Pretreatment of biomass and hydrolysis of cellulose and hemicellulose followed by sugar fermentation have several drawbacks including the cost of pretreatment and enzymes, which makes the process more expensive than traditional wet and dry corn milling processes. Excessive treatment of biomass with acid and high temperature can degrade the released sugar molecules to furfural and hydroxymethyl furfural. These in turn can inhibit the microorganisms used to produce ethanol and can degrade to undesired products such as tars (Wyman 1994). Additional drawbacks include the recalcitrant nature of biomass, need for genetically engineered microorganisms to convert lignocellulosic sugars to ethanol and high transportation costs of low density biomass makes the bioconversion of lignocellulosic material to ethanol through hydrolysis-fermentation difficult (Balat 2008). The hydrolysis and fermentation process is still under research and there are very few companies established with this technology. Few pilot scale plants have been established and research is being focused to scale up the process.

There is a need to explore new technologies that can efficiently convert biomass to fuels. Gasification-fermentation is a hybrid technology in which any carbon-based

feedstock can be converted to ethanol using a suitable microbial species (Reed 1981). Gasification is a controlled pyrolysis and reduction process in which all fractions of the biomass, including lignin, are converted to synthesis gas composed of CO, CO_2, H_2, N_2, and residual tars (Datar et al. 2004; Demirbas et al. 2004). Synthesis gas can then be converted to ethanol and other value added products using acetogens such as *Clostridium ljungdahlii* (Cotter et al. 2009), *Clostridium autoethanogenum* (Abrini et al. 1994) and *Clostridium carboxidivorans* ($P7^T$) (Rajagopalan et al. 2002). Although the biological process of conversion of synthesis gas to ethanol is slow, it is considered to be advantageous compared to catalytic conversion, as it is associated with mild operational conditions and reduced capital costs (Worden et al. 1991). *Clostridium* strain, P11 is used in the current research. Strain P11 has the ability to grow autotrophically on CO/CO_2 or H_2/CO_2 mix and produce acetate and ethanol (Huhnke et al. 2008).

The primary objective of this study is to compare the growth and product kinetics of *Clostridium* strain P11 during syngas fermentation.

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CHAPTER II

LITERATURE REVIEW

2.1 Introduction

Plant biomass was used as a major energy resource before the discovery of fossil fuels. The discovery of inexpensive crude oil in the 19th century led to an industrial revolution of western countries. This helped these countries to develop their infrastructure and improve the standards of living for their citizens (Huber et al. 2006). In the United States, about half of the gasoline consumed by the transportation and industrial sector is imported (Demain et al. 2005). Fossil fuels are finite resources, fluctuate in price, and impact the environment. Thus, there is a need to develop sustainable liquid fuels such as ethanol from renewable resources such as plant biomass (Huber et al. 2006). These liquid fuels are more environmentally friendly but require improvements in order to make them economical and energy efficient.

Carbon dioxide is the primary green house gas (GHG) emitted by human activities, contributing to 85% of the total GHG emissions. Combustion of fossil fuels is the major source of CO_2 (Renewable Fuels Association 2009). Since, the transportation sector is limited to liquid fuels; it is preferable to use biomass as a feedstock for the production of liquid biofuels. Biofuels could significantly reduce GHG emissions from vehicles and also promote the economic status of the country (Balat and Balat 2009).

Ethanol is a clean energy source which on combustion generates the same amount of CO_2 as plants utilize during photosynthesis (Goldemberg et al. 2008). Ethanol is biodegradable and soluble in water, which makes it a preferable additive to gasoline than methyl tertiary butyl ether (MTBE). Also, MTBE is proved to be a carcinogen and contaminant to underground water (Nadim et al. 2001). The incorporation of ethanol into gasoline at various fractions increases the octane number and hence the activation energy required to ignite an engine. Currently in the U.S., ethanol is blended with gasoline primarily at two proportions, namely E10 (10% ethanol and 90% gasoline) and E85 (85% ethanol and 15% gasoline). Vehicle modification is necessary to incorporate E85 as a fuel (Demirbas 2008). A study by the National Center for Vehicle Emissions Control and Safety at Colorado State University found that the use of E10 as a fuel in the internal combustion engine reduced CO emissions by 25-30% (Goldemberg et al. 2008).

Advantages of ethanol as a fuel include:

- Has higher octane number, broader flammability limits, higher flame speeds and higher heats of vaporization.
- Can be produced from domestically available renewable resources.
- Is environmentally friendly and biodegradable.
- Can contribute to green house gas reductions.
- Can create jobs in rural industries.
- Provides higher combustion efficiency.

Disadvantages of ethanol as a fuel include:

- Has a low energy density compared to gasoline.
- Is corrosive in nature.

- Has a lower vapor pressure making cold starts difficult.
- Releases evaporative emissions when blended with gasoline.
- Raw materials constitute 60-75% of the final fuel cost; therefore, it is very important to develop efficient and feasible conversion technologies to produce sustainable fuels.

2.2 Raw materials for ethanol production and their economical impact

Feedstocks used for ethanol production can be divided into three groups (Balat and Balat 2009):

- Sugar containing feedstocks such as sugarcane, sugar beet and sweet sorghum.
- Starch containing materials like corn, wheat, barley, rice, and potatoes.
- Lignocellulosic biomass such as agricultural residues such as corn stover, forest residues such as wood, grasses and aquatic biomass such as algae.

In the United States, corn is predominantly used as the major feedstock for ethanol production. In order to meet the increasing demand of ethanol, large proportions of high-quality land must be used for the cultivation of energy crops such as corn, wheat, sugarcane, and soybeans (Luchansky and Monks 2009). The utilization of corn for ethanol production in the U.S. is expected to rise from 12% to 23% by 2015 (Runge and Senauer 2007). The increasing demand for corn towards ethanol production might have led to inflation of its price from $2/bushel to more than $4/bushel by early 2007 (Renewable Fuels Association 2007). The increasing prices of corn and other food grains directly affect the food industry. Staple grains, to some extent, could replace corn demand in the food sector, but the price of staple grains also increased as many farmers preferred to grow corn over other crops. This increased the pressure on other edible crops and

grains. Corn is used in the production of various goods ranging from high fructose corn syrup to cattle feed. Meat and other livestock production became more expensive with the increase in grain prices. Farmers diverted from soybean cultivation to corn to make profits from the increasing demand for corn in markets such as ethanol and food industries. This led to the increased price of popular trans-fat-free cooking oil (Rosenwald 2007). In order to increase the yield of corn, more land must be brought under cultivation. Land would be available either by deforestation or by converting grasslands into corn (Rathmann et al. 2010). The competition for land between food and biofuels can be reduced by the following factors (Goldemberg et al. 2008; Leemans et al. 1996; Pimentel and Patzek 2007; Turpin et al. 2009):

- Develop and implement efficient technologies to convert cellulosic material to ethanol.
- Good management practices should be used for production of biomass feedstocks or energy crops, harvesting, storage and transportation of biomass to biorefinery.
- Increased use of marginal and pasture lands for cultivation of cellulosic feedstock and utilization of lands rich in carbon for cultivation of food crops.

According to Hammerschlag (2006), 60-75% of the energy present in corn ethanol is consumed during its production. In other words, about 20-25% of the energy is utilized to cultivate and harvest corn, 40-50% is consumed during conversion of corn to ethanol. However, corn alone cannot support the ethanol demand over the next several years. Therefore, it is necessary to develop new technologies to increase the yield of ethanol from available biomass resources.

Ethanol can also be produced from renewable raw materials such as herbaceous and woody biomass, agricultural residues, and municipal solid, forestry, and other biological wastes. Lignocellulosic crops such as switchgrass are abundant and can be cultivated in low-quality lands and require less energy inputs, fertilizers, water, and nutrients supply (Hamelinck et al. 2005; Huber et al. 2006).

2.3 Ethanol production processes

Most of the ethanol in the US is produced through fermentation of carbohydrates from starch and sugar crops. The biofuels industry is still in its infancy and extensive research is being focused on developing novel conversion technologies to produce ethanol from inexpensive agricultural crops and residues (Demirbas 2005; Huber et al. 2006).

2.3.1 Ethanol from corn

Corn based ethanol is produced through well known traditional technologies known as dry milling (grinding process) and wet milling (chemical extraction process). The basic steps in the conversion process of corn to ethanol are as follows:

- Starch is extracted from corn either by dry milling or wet milling processes.
- Starch is then converted to glucose by enzymatic treatment, followed by fermentation of glucose to ethanol by yeast.
- Pure ethanol is separated from the fermented broth by distillation.
- Finally, ethanol is denatured by the addition of additives such as methanol, acetone, denatonium, wood naphtha to make it unfit for human consumption (European-Union 1993).

In addition to ethanol production, there are four major co-products from the wet-milling process, which are condensed corn fermented extractives or corn steep liquor, corn germ meal, corn gluten feed and corn gluten meal. The major co-products from dry-milling process are corn condensed distillers solubles and corn distillers dried grains with soluble (DDGS). Corn ethanol is mainly limited to the mid western United States which are rich in corn production. Transportation costs of ethanol to other states would increase the fuel price at the pump. Therefore, it is economically feasible to construct ethanol plant near the corn belt. However, corn alone cannot meet the future ethanol demand, so it is necessary to look for cheap alternative biomass resources.

2.3.2 Ethanol from lignocellulosic feedstocks

Lignocellulosic plants can be cultivated in less fertile and low cost lands. These crops require low energy inputs, fertilizers and water inputs. Lignocellulosic biomass is mainly composed of cellulose (40-60%), hemicellulose (20-40%) and lignin (10-25%) (Hamelinck et al. 2005). As shown in Figure 2.1, lignin is a large polyaromatic component that forms the cell walls and provides a rigid support to the cells. Cellulose is the main part of the plant body and is a linear polymer of anhydroglucose molecules connected by β-1-4-glycosidic bonds. Linear chains of cellulose form a rigid crystalline structure and the hydrogen bonds between the chains make the complex structure rigid and difficult to break (Hamelinck et al. 2005; Lynd 1996). Hemicellulose is also a polysaccharide with monomer units that include pentoses (arabinose and xylose), hexoses (glucose, galactose, mannose, rhamnose and fucose) and uronic acids (galacturonic, glucoronic and methylglucuronic acid). Hemicellulose can be easily degraded compared to cellulose because of its branched structure.

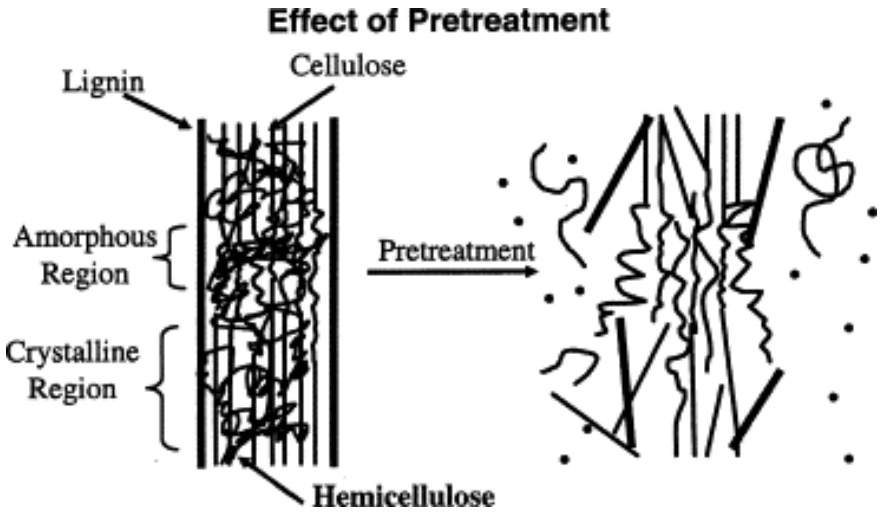

Figure 2.1 Effect of pretreatment of lignocellulosic material (Mosier et al. 2005).

The depolymerization of cellulose molecules is very difficult because of its rigid crystalline structure (Lange 2007). Pretreatment techniques break lignocellulosic material and increase the accessibility of cellulose to enzymes. The process of ethanol production from lignocellulosic material consists of four major steps: pretreatment, hydrolysis, fermentation and product recovery as shown in Figure 2.2 (Mosier et al. 2005).

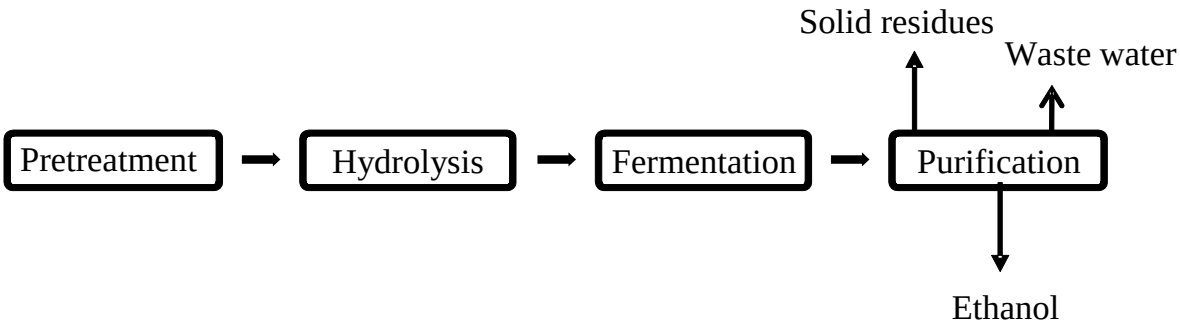

Figure 2.2 An overview of ethanol production from biomass through hydrolysis process adopted from (Hamelinck et al. 2005).

Step 1. Pretreatment: This step is required to break the complex structure of lignocellulosic material and make cellulose more accessible to enzymes; thereby, increasing the yield of sugars during hydrolysis, which are further fermented to ethanol. This step reduces the sample size, softens the biomass, and breaks the rigid cell structure; thereby, making it easy for enzymes and acids in the hydrolysis step to access cellulose (Fatih Demirbas 2009). Pretreatment of biomass can increase the yield of cellulose hydrolysis to as high as 90% (Balat and Balat 2009). According to Mosier et al (2005), a successful pre-treatment method should meet the following requirements:

- Pretreatment must promote higher yields of sugars and products in the hydrolysis and fermentation steps.
- Degradation or loss of carbohydrates should be avoided or reduced.
- Formation of intermediate products that inhibit and decrease yields in the subsequent hydrolysis and fermentation steps should be avoided.
- Process should be cost effective.

Overall, pretreatment is important in increasing the yield of ethanol. Before the addition of enzymes in hydrolysis, the prehydrolysate must be conditioned (i.e., the temperature and pH have to be adjusted to the optimum range at which the enzymes activities are high).

Pretreatment methods can be classified into chemical or physical type based on the reagent or machine used. Various pretreatment methods are listed in Table 2.1.

Table 2.1 Pretreatment methods under evaluation (Pienkos and Zhang 2009).

Pretreatment category	**Specific pretreatment technology**
Base catalyzed	- AFEX/FIBEX(*Ammonia fiber explosion/ fiber extrusion explosion)* - Ammonia recycle percolation - Lime
Non catalyzed (mechanical)	- Autohydrolysis-steam - Hot water - Hot water pH neutral
Acid catalyzed	- Carbonic acid - Nitric acid - Sulfuric acid
Solvent based	- Organosolv
Chemical based	- Wet oxidation (O_2, peroxide, ozone)

Step 2. Hydrolysis: The purpose of hydrolysis is to convert the cellulose and hemicellulose to simple sugars (monomeric sugars). This can be achieved either using chemicals (concentrated acid or dilute acid) or enzymes. Cellulose can be hydrolyzed to glucose either by cellulase or chemically by sulfuric acid or any other acids. Moreover, hemicellulases or acid hydrolysis can catalyze the release of simple sugars such as xylose and arabinose from hemicellulose.

Step 3. Fermentation: Six carbon sugars such as glucose, galactose and mannose are easily fermented to ethanol by many naturally available microorganisms. There are only few native strains available to ferment pentoses, i.e. five carbon sugars, such as xylose and arabinose to ethanol resulting with low yields. Genetically modified strains are being developed to ferment pentoses to ethanol. Hydrolysis and sugar fermentation from biomass feedstocks have drawbacks, such as complex pretreatment and costly enzymes make the process more expensive than traditional wet and dry corn milling process. Excessive treatment of sugars with acid or high temperature degrade sugars to furfural and hydroxymethyl furfural. These in turn can degrade to undesired products such as tars (Wyman 1994).

Research is being carried out towards process integration in order to reduce production costs by reducing the number of unit operations or reactors required to produce ethanol (Wright et al. 1988). The most common processes are:

- Separate hydrolysis and fermentation (SHF): process in which enzymatic hydrolysis and fermentation are carried out in two different reactors.
- Simultaneous saccharification and fermentation (SSF): cellulose hydrolysis is conducted in the presence of fermentative microorganism in the same tank.
- Simultaneous saccharification and co-fermentation (SSCF): It is similar to SSF with co-fermentation of both glucose and xylose either by genetically modified strains or by using co-cultures.

On an industrial basis, SSF and SSCF are desirable because the process can be limited to a single container which would further reduce the capital costs (Wright et al. 1988).

Step 4. Product separation or purification: Ethanol and other by-products produced during the fermentation are recovered by distillation of the fermentation broth.

Various factors such as the recalcitrant nature of biomass and the need for genetically engineered microorganisms to convert hexoses and pentoses to ethanol makes the bioconversion of lignocellulosic material to ethanol through hydrolysis-fermentation difficult (Balat 2008).

2.3.3 Ethanol production by gasification-fermentation process

Gasification-fermentation is a hybrid technology in which any carbon-based feedstock can be converted into ethanol using a suitable microbial species (Reed 1981). It is a two stage process. In the first stage biomass is gasified to produce synthesis gas (syngas) and in the second stage microbial catalysts are utilized to convert the syngas into ethanol and other value added products.

2.3.3.1 Gasification of biomass to synthesis gas

Gasification is a thermochemical process that converts all biomass components, including lignin, to synthesis or producer gas composed of CO, CO_2 and H_2 with small amounts of impurities. Gasification converts any carbon-based feedstock to combustible gases, which either can be burned to generate electricity or converted to fuel ethanol through various processes (Bauen 2004). Ethanol can be produced from syngas either through chemical catalysis or biological processing. Compared to chemical catalysis, biological processing for ethanol production from syngas is slow. However, biological processing is more efficient and has potential economic benefits compared to chemical catalysis process because the former method requires a mild operating temperature and pressure (Worden et al. 1991). In addition, the gasification-fermentation process is

advantageous to the biochemical platform (hydrolysis-fermentation process) because only one biological step (i.e. fermentation of syngas) is required (Phillips et al. 1994).

During gasification of biomass, the gasifiers should be operated at temperatures over 1000°C to achieve the greatest yields of CO and H_2 (Kasteren et al. 2005). Yields of gases from the gasification of biomass depend on three main parameters: type of gasifier, operating conditions of the gasifier and type of gasifying agent used. Common gasifying agents are steam, air and oxygen. Partial oxidation of biomass with steam under oxygen-limited conditions produces syngas with high CO and H_2. Typical syngas composition under these conditions is 40-65% CO, 1-20% CO_2, 25-35% H_2 and 0-7% CH_4 (Gupta and Cichonski 2007). Partial oxidation of biomass under similar conditions with air generates producer gas with a different composition (15% CO, 10-15% CO_2, 15-20% H_2 and 40-50% N_2). Gasification with pure oxygen is expensive, so air is normally used as a gasifying agent. But the disadvantage of using air is that N_2 (79%) present in air dilutes the final syngas and reduces the yields of other gases (McKendry 2002).

The thermochemical reactions that occur in the gasifier during conversion of biomass to synthesis gas are summarized in Table 2.2. Equations 2.1 to 2.3 are oxidation reactions and the gasifying agent either can be air or steam. Also, CO and steam react further to form H_2 via the water gas shift reaction (Equation 2.4) and CO and H_2 react to produce methane (Equation 2.5).

The major disadvantage of the gasification process is the formation of tars and other higher hydrocarbons, which can inhibit cell growth (Ahmed and Lewis 2007). Also, producer gas can contain traces of nitric oxide, ammonia, and cyanide. Tar formation can

be reduced to a certain extent by operating the gasifier at temperatures greater than 1000°C (Kasteren et al. 2005).

Table 2.2 Thermochemical reactions and heats of reaction (ΔH) that take place in the gasifier (McKendry 2002).

Reaction	Equation	ΔH (MJ/kg mole)	No.
Partial oxidation	$C+1/2O_2 \leftrightarrow CO$	-110.5	2.1
Complete oxidation	$C + O_2 \leftrightarrow CO_2$	-406	2.2
Water-gas reaction	$C + H_2O \leftrightarrow CO + H_2$	+131.3	2.3
Water-gas shift	$CO + H_2O \leftrightarrow CO_2 + H_2$	-41.1	2.4
Methane formation	$CO + 3H_2 \leftrightarrow CH_4 + H_2O$	-206.1	2.5

2.3.3.2 Fermentation of synthesis gas using microbial catalyst

Anaerobic microorganisms such as acetogens and methanogens could serve as catalysts for the conversion of inorganic substrates such as CO, CO_2 and H_2 into fuel (Najafpour and Younesi 2006). Acetogens are found to be capable of metabolizing single-carbon compounds to produce ethanol and other high molecular weight products via acetogenic fermentation (Worden et al. 1991). Syngas can be metabolized to ethanol and butanol by several microbial catalysts (Vega et al. 1989b; Worden et al. 1991). The stoichiometry for ethanol and acetic acid production from syngas is (Vega et al. 1989b):

$$6CO + 3H_2O \rightarrow C_2H_5OH + 4CO_2 \quad (2.6)$$

$$6H_2 + 2CO_2 \rightarrow C_2H_5OH + 3H_2O \quad (2.7)$$

$$4CO + 2H_2O \rightarrow CH_3COOH + 2CO_2 \quad (2.8)$$

$$2CO_2 + 4H_2 \rightarrow CH_3COOH + 2H_2O \quad (2.9)$$

The above stoichiometry does not account for carbon assimilated into cell mass. If CO is used as a sole source of carbon and energy, then one-third of carbon from CO can theoretically be converted to ethanol and the remaining two-thirds to CO_2 (Equation 2.6). When equal moles of CO and H_2 are supplied, theoretically, two-thirds of the carbon from CO should be converted to ethanol and the remaining is accounted for in CO_2 (Equation 2.10) (Rajagopalan et al. 2002).

$$3CO + 3H_2 \rightarrow C_2H_5OH + CO_2 \quad (2.10)$$

When Equations 2.8 and 2.9 are combined, all the carbon supplied in the form of CO should theoretically be converted to acetic acid (Equation 2.11).

$$2CO + 2H_2 \rightarrow CH_3COOH \quad (2.11)$$

Butanol can also be produced from syngas and the stoichiometry is:

$$12CO + 5H_2O \rightarrow C_4H_9OH + 8CO_2 \quad (2.12)$$

$$12H_2 + 4CO_2 \rightarrow C_4H_9OH + 7H_2O \quad (2.13)$$

2.4 Acetogens

Acetogens can metabolize single-carbon substrates such as CO and CO_2 to acetate and ethanol (Zeikus et al. 1985). Few anaerobic bacteria have the ability to ferment sugars to ethanol, acetate, CO_2 and H_2 (Andreesen et al. 1989). *Butyribacterium methylotrophicum* can grow on a wide variety of substrates such as glucose, formate, H_2, CO, CO_2 and produce acetic acid, butyric acid, ethanol and butanol (Rogers 1986). Few *Clostridium* species, such as *Clostridium ljungdahlii* (Phillips et al. 1994), *Clostridium*

autoethanogenum (Abrini et al. 1994),and *Clostridium carboxidovorans* P7 (Rajagopalan et al. 2002), can grow on syngas components and yield ethanol and acetic acid.

Clostridium ljungdahlii is the first known autotrophic microorganism to produce ethanol and acetate from bottled syngas (Klasson et al. 1992b). Using *Clostridium ljungdahlii* for syngas fermentation, a maximum of 48 g/L ethanol and 3 g/L acetate were produced after 560 h of fermentation in a continuous stirred tank reactor (CSTR) coupled with a cell recycle system. The conversion efficiencies of CO and H_2 after 500 h of fermentation were 90 and 70%, respectively (Klasson et al. 1993)

Clostridium autoethanogenum was found to metabolize syngas as well as other carbon substrates including xylose, pyruvate, glutamate and rhamnose to produce ethanol, acetate, H_2 and CO_2 (Abrini et al. 1994).

Clostridium carboxidivorans P7 is a novel anaerobic bacterium that demonstrated the ability to produce ethanol, acetic acid and butanol when grown on CO, CO_2, and H_2 (Ahmed and Lewis 2007; Datar et al. 2004; Liou et al. 2005; Rajagopalan et al. 2002). Besides indirect fermentation of biomass syngas to ethanol, P7 has the capability to catalyze direct conversion of lignocelluloses to ethanol and acetate. P7 catalyzed the conversion of 100 mmol fructose to 23 mmol ethanol, 81 mmol acetate and 4 mmol butanol. With an equivalent amount of carbon monoxide, i.e. 600 mmol, the concentrations of end products were 96 mmol ethanol, 12 mmol acetate and 24 mmol butanol (Liou et al. 2005). A reducing environment is necessary for anaerobic metabolism to produce ethanol, so more ethanol is produced from CO because of its reducing nature (Klasson et al. 1992a).

Clostridium strain P11 is gram positive, rarely motile, rod shaped bacteria and can occur singly or in chains. It has the ability to grow autotrophically on CO/CO_2 or H_2/CO_2 mix. Also it is known to grow chemoorganotrophically on several substrates such as glucose and fructose to produced ethanol and acetate. The optimum temperature and pH are 37°C and 6.1, respectively (Huhnke et al. 2008).

Methanogens and acetogens utilize the reductive acetyl-CoA pathway, which is also known as Wood – Ljungdahl pathway, to grow on single carbon substrates such as CO and CO_2. *Clostridium* bacteria use either H_2 or organic compounds as electron sources for the reduction of CO_2 to acetyl-CoA, which is further converted to acids and alcohols (Wood et al. 1986).

2.4.1 Flow of carbon in Wood-Ljungdahl or reductive acetyl-CoA pathway

Acetogenic bacteria grow autotrophically and use CO, CO_2 and H_2 as a sole source of carbon and energy. Wood and Ljundhal defined acetogenesis as a process of conversion of two CO_2 molecules to one acetate molecule (Drake 1992). Acetogenic bacteria utilize the autotrophic pathway known as Wood-Ljungdahl or acetyl-CoA pathway for its growth on CO, CO_2 and H_2 (Wood et al. 1986). Acetyl-CoA serves three main purposes for acetogens (Drake 1994):

- An electron sink under heterotrophic conditions.
- Energy conservation in the reductive direction.
- A mechanism for the autotrophic assimilation of carbon.

The acetyl-CoA pathway can occur in both the oxidation and reduction directions. In the oxidative direction, acetate is metabolized to CO_2 and, in the reductive direction, CO_2 is reduced to acetate (Ragsdale 1997). Acetogens conserve energy by the reduction

of CO_2 and H_2 to acetate. Homoacetogens depend on the acetyl-CoA pathway for the production of acetate, ethanol, butyrate and butanol. Acetyl-CoA serves as a precursor for cellular carbon (assimilation) and reduced products (excretion). It also links the catabolic and anabolic pathways (Eden and Fuchs 1983).

In the acetyl-CoA pathway, CO_2 is reduced to acetyl-CoA through two distinct branches, namely "methyl branch" and "carbonyl branch" as shown in Figure 2.3. An acetyl-CoA synthase/carbon monoxide dehydrogenase complex (ACS/CODH) catalyzes the condensation of a carbonyl and a methyl group with coenzyme A (CoA) to form acetyl-CoA (Lindahl 2002; Ragsdale 2004). In the carbonyl branch, CODH reduces CO_2 to a carbonyl group [CO], and in the methyl branch, CO_2 is reduced to methyl-tetrahydrofolate (CH_3-THF), which is reduced further by tetrahydrofolate (THF) to a methyl corrinoid protein or enzyme (Drake et al. 1981). The cobalt-containing corrinoid enzyme helps in the transfer of the methyl group from CH_3-THF to the C_2 position of acetate (Hu et al. 1984).

As shown in Figure 2.3, the acetyl-CoA pathway utilizes eight reducing equivalents or electrons to reduce CO_2 to acetate (Equation 2.14).

$$2CO_2 + 8H^+ + 8e^- \rightarrow CH_3COOH + 2H_2O \quad (2.14)$$

Carbon monoxide dehydrogenase (CODH) and hydrogenase enzymes play an important role in supplying reducing equivalents for the reduction of CO_2 to acetyl-CoA (Ljungdhal 1986). If a mixture of CO, CO_2 and H_2 is used for fermentation, CODH catalyzes the oxidation of CO to CO_2 (Equation 2.15) and generates reducing equivalents necessary for the reduction of CO_2 to acetyl-CoA (Ljungdhal 1986).

$$CO + H_2O \leftrightarrow CO_2 + 2H^+ + 2e^- \quad (2.15)$$

Six reducing equivalents are utilized in the methyl branch for the reduction of CO_2 to acetyl CoA, while the carbonyl branch requires two electrons for the reduction of CO_2 to carbonyl chain intermediate [CO] (Ljungdhal 1986). The CODH is not necessary if CO is readily available for the formation of precursor in the carbonyl branch (Svetlitchnyi et al. 2004). The reducing equivalents necessary for the reduction of CO_2 to acetyl-CoA are either supplied by the oxidation of CO by CODH (Equation 2.15) or by the oxidation of hydrogen catalyzed by non nickel containing hydrogenase (Equation 2.16).

$$H_2 \leftrightarrow 2H^+ + 2e^- \quad (2.16)$$

Previous studies have shown that non nickel containing hydrogenases are present in *Acetobacterium woodii* (Diekert and Ritter 1982) and *Clostridium thermoaceticum* (Martin et al. 1983). However, if H_2 present in the syngas is used to generate electrons, then more CO can be utilized towards cell growth and products formation (Menon and Ragsdale 1996). CODH can catalyze both reduction of CO_2 and oxidation of CO. Therefore, both CO and CO_2 can be used for the production of ethanol by acetogens. The detailed mechanisms for formation of acetyl-CoA via the methyl and carbonyl branches are discussed next.

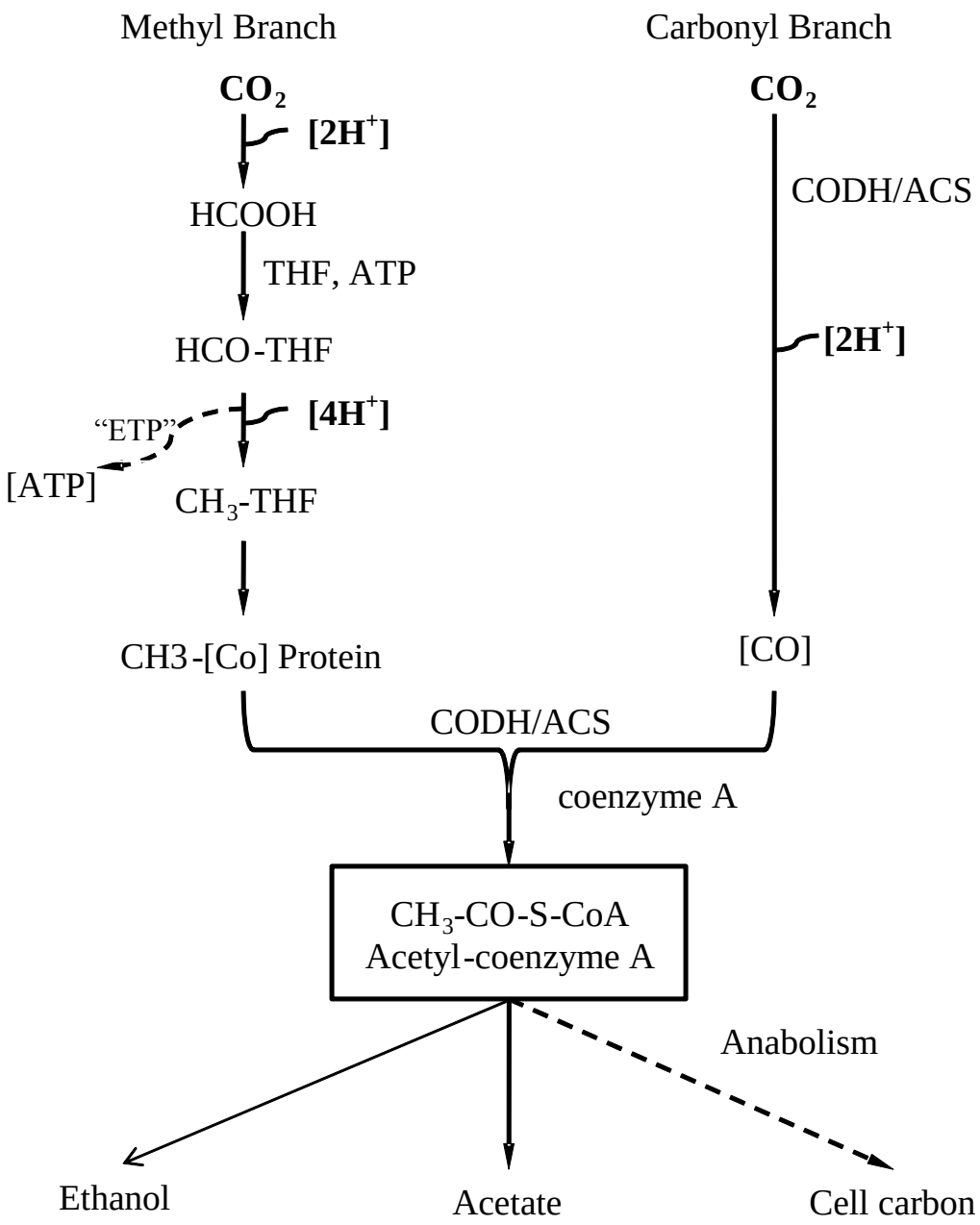

Figure 2.3 Simplified schematic of the Wood-Ljungdahl pathway of acetogens for ethanol and acetate production from carbon dioxide. THF-tetrahydrofolate, ACS-Acetyl-CoA synthase, [Co] protein-corrinoid enzyme, ETP- Electron Transport Phosphorylation (Drake 1994).

2.4.2 The methyl branch of acetyl-CoA pathway

Various enzymes are involved in the conversion of CO_2 to acetyl-CoA in the methyl branch. The first reaction in the production of acetyl-CoA from CO_2 is a two electron reduction of CO_2 to formate (HCOOH), which is catalyzed by formate dehydrogenase.

$$CO_2 + 2H^+ + 2e^- \rightarrow HCOOH \quad (2.17)$$

Depending on the type of bacteria, the electron donor can be either pyridine nucleotides or ferredoxin (Diekert and Wohlfarth 1994a). Then, formyl-tetrahydrofolate synthetase catalyzes the condensation of formate with tetrahydrofolate (THF) to form formyl-H_4folate at an expense of one adenosine triphosphate (ATP) molecule that is converted to adenosine diphosphate (ADP).

$$HCOOH + ATP + H_4folate \rightarrow 10\text{-}HCO\text{-}\ H_4folate + ADP + Pi \quad (2.18)$$

After which, methenyl-THF cyclohydrolase catalyzes the dehydration of 10-formyl- H_4folate to 5, 10 -methenyl -H_4folate.

$$10\text{-formyl-}\ H_4folate + H \rightarrow 5,\ 10\text{ -methenyl -}H_4folate + H_2O \quad (2.19)$$

Then, 5, 10 -methenyl -H_4folate is further reduced to 5, 10-methylene –H_4 folate by 5, 10-methylene tetrahydrofolate dehydrogenase. In this reaction, the C_1 unit is transferred from formate to further reduced formaldehyde.

$$5,\ 10\text{ -methenyl -}H_4folate + NAD(P)H \rightarrow 5,10\text{-methylene –}H_4\text{ folate} + NAD(P)^+ \quad (2.20)$$

The 5, 10-methylene –H_4 folate is catalyzed by 5, 10-methylene tetrahydrofolate reductase to form 5-methyl H_4 folate.

$$5,\ 10\text{-methylene –}H_4\text{ folate} + 2H + 2e^- \rightarrow 5\text{-methyl }H_4\text{ folate} \quad (2.21)$$

After the above reaction, the methyl unit in 5-methyl H_4 folate is transferred to reduced cobalt in the corriniod protein or enzyme. This reaction is catalyzed by the enzyme methyltransferase.

$$\text{5-methyl } H_4 \text{ folate} + \text{E-[Co]} \rightarrow \text{FH4} + \text{E-[Co]-}CH_3 \quad (2.22)$$

It is theorized that there is an ATP generation by a chemiosmotic mechanism in the above reaction because the standard redox potential for the methylene/methyl tetrahydrofolate is found to be around -200 mV, which is sufficiently exergonic for the formation of ATP (Diekert and Wohlfarth 1994a; Wohlfarth and Diekert 1991).

2.4.3 The carbonyl branch of acetyl-CoA pathway

The carboxyl group of acetate can be derived from CO_2 through the carbonyl branch of acetyl-CoA pathway (Diekert and Wohlfarth 1994a). Ni containing CODH or acetyl-CoA synthase catalyzes the reduction of CO_2 to [CO], which forms the intermediate in the carbonyl chain (Diekert and Thauer 1980; Diekert et al. 1979; Drake 1994).

$$CO_2 + 2H^+ + 2e^- \rightarrow [CO] + H_2O \quad (2.23)$$

Finally, the carbonyl molecule [CO] from the carbonyl chain merges with the methyl group from the methyl chain to form an acetyl-CODH moiety. CODH, also known as acetyl-CoA synthase, catalyzes the condensation of the acetyl moiety with free coenzyme-A to form acetyl-CoA.

$$\text{E-[Co]-}CH_3 + [CO] \rightarrow \text{E-[Co]} + \text{Acetyl-CoA} \quad (2.24)$$

Acetyl-CoA is an important metabolite in the acetyl-CoA pathway and is also the precursor for the formation of lipids, amino acids, nucleotides and carbohydrates

(Ljungdhal 1986). Acetyl-CoA goes through the anabolic pathway and is reductively converted to phosphoenolpyruvate, which forms cellular material (Diekert and Wohlfarth 1994b). Acetyl-CoA can also be converted to acetate, butyrate, ethanol and butanol (Figure 2.4). Acetogens conserve energy through the acetyl-CoA pathway. Through the catabolic pathway, acetyl-CoA forms acetate and ATP. The latter is necessary for cell growth, therefore acetate is produced during growth conditions, a process known as acidogenesis.

In the acidogenic phase, phosphotransacetylase catalyzes the conversion of acetyl-CoA to acetyl-phosphate by removing CoA and adding phosphate to the acetyl group (Equation. 2.25). This reaction is followed by conversion of acetyl-phosphate to acetate with the release of ATP by phosphorylation of ADP (Equation 2.26). Moreover, under slow growth or non-growth conditions, solvents are produced and this process is known as solventogenesis (Rao and Mutharasan 1989). During the solventogenic phase, there is no evolution of ATP because cells are not in the active growth phase. In the solventogenic phase, acetaldehyde dehydrogenase catalyzes the conversion of acetyl-CoA to acetaldehyde and this reaction depends on reducing power supplied by the oxidation of NADH (Equation 2.27).

$$\text{Acetyl- CoA} + P_i \rightarrow \text{Acetyl-phosphate} + P_i \quad (2.25)$$

$$\text{Acetyl-phosphate} + \text{ADP} \rightarrow \text{Acetate} + \text{ATP} \quad (2.26)$$

$$\text{Acetyl-CoA} + \text{NADH} + H^+ \rightarrow \text{acetaldehyde} + \text{NAD}^+ + \text{CoA} - \text{SH} \quad (2.27)$$

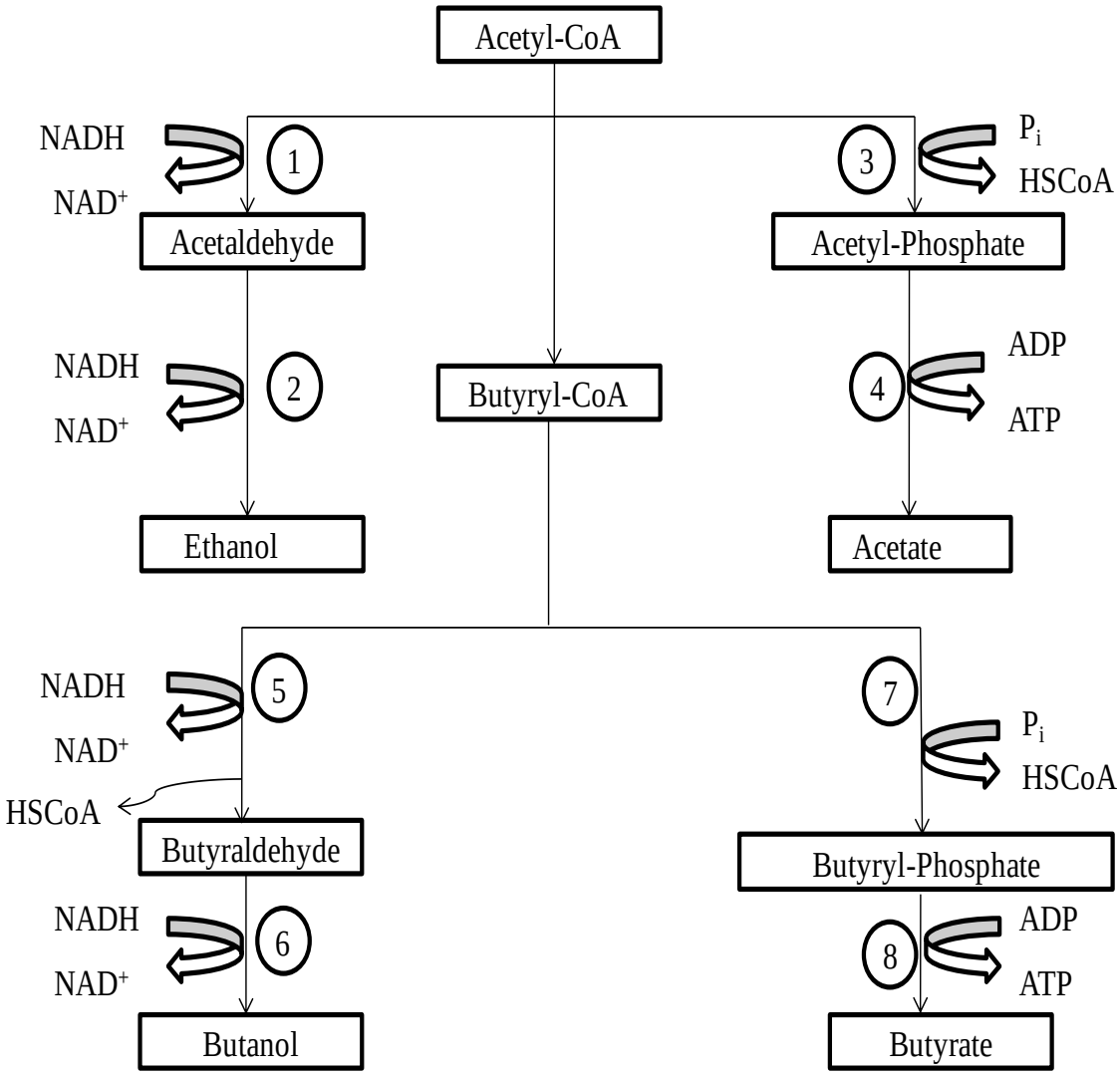

Figure 2.4 The anabolic and catabolic role of acetyl-CoA in *Clostridium acetobutylicum* adapted from (Vasconcelos et al. 1994). 1-acetaldehyde dehydrogenase, 2-alcohol dehydrogenase, 3- phosphotransacetylase, 4-acetate kinase, 5-butyraldedyde dehydrogenase, 6-butanol dehydrogenase, 7-phosphotransbutyrylase, 8-butyrate kinase.

Finally, alcohol dehydrogenase catalyzes the conversion of acetaldehyde to ethanol using reducing power from the oxidation of NADH (Equation 2.28).

$$\text{Acetaldehyde} + \text{NADH} + H^+ \rightarrow \text{Ethanol} + NAD^+ \quad (2.28)$$

Therefore, during syngas fermentation, acetic acid production can be seen during growth conditions and ethanol formation starts under non-growth conditions. Higher alcohols like butanol are also produced through the acetyl-CoA pathway. Two molecules of acetyl-CoA combine to form an acetoacetyl-CoA intermediate, which functions similarly to acetyl-CoA (Rao and Mutharasan 1987).

Factors like pH, sporulation, ATP levels, electron flow, and availability of nutrients and reducing equivalents (reducing power) effect solventogenesis (Adler and Crow 1987; Dürre et al. 1995; Girbal et al. 1995; Gottschal and Morris 1981; Guedon et al. 1999; Meyer et al. 1986; Vasconcelos et al. 1994).

2.4.4 Energetics of Acetyl-CoA pathway

In the reductive acetyl-CoA pathway, one mole of ATP is invested to activate formate, which is further reduced to formyl-H_4folate with the consumption of ATP (Equation 2.18). Formyl-H_4folate is further reduced to acetyl-CoA. Therefore, the formation of acetyl-CoA from syngas has a negative energy balance. This negative energy in the metabolic pathway is balanced by ATP production through substrate level phosphorylation in the acetate kinase reaction during the formation of acetate (Equation 2.26) (Muller 2003). Therefore, the net ATP balance through substrate level phosphorylation in the acetyl-CoA pathway is zero. It was shown that acetogens generate ATP through electron transport phosphorylation (ETP) linked to the dehydrogenative (CODH) and hydrogenative (THF-linked reductions) reactions (Zeikus et al. 1985).

Various electron carriers are found in acetogens either in soluble form (ferredoxin, flavodoxin, and rubredoxin) or as membrane bound (cytochrome b and menaquinone) (Gottwald et al. 1975).

2.5 Advantages and disadvantages of syngas fermentation

The advantages of syngas fermentation are:

- Biological conversion occurs at low temperature and pressures compared to the catalytic syngas conversion process (Worden et al. 1991).
- Higher specific activity of microorganism results in higher productivity of desired products; hence, reduces the cost of product recovery (Vega et al. 1989a; Worden et al. 1991).
- Microorganisms are tolerant to contaminants in syngas such as sulfur gases; hence, extensive cleaning of syngas prior to conversion step is not needed (Ahmed and Lewis 2007; Vega et al. 1990).
- Biological conversion does not require a set ratio of CO/H_2. Thus, any kind of biomass or waste can be used as a raw material (Huber et al. 2006).
- Gasification process converts whole fraction of biomass into syngas including lignin (Phillips et al. 1994).
- Most of the biological reactions are irreversible and complete conversion of biomass can be achieved by using suitable microorganisms (Najafpour and Younesi 2006).

Apart from all the above advantages, syngas fermentation technology has some drawbacks such as:

- The metabolic energy produced by acetogens when grown on syngas is very low and this results in slow growth, low cell density and solvent production during non-growth phase (Tsai et al. 2009a).
- Low solubility of CO and H_2 in the medium makes the fermentation process mass transfer limited (Vega et al. 1990).
- Slow reactions rates during syngas fermentation result in high residence times (Vega et al. 1989a).
- Product recovery is expensive because of the dilute nature of the products in the outlet stream (Vega et al. 1989a).

The rate limiting step in the fermentation of syngas is the mass transfer from the gas phase to the microorganism in the liquid phase (Vega et al. 1990; Worden et al. 1997). It is necessary to increase the mass transfer rate of CO and H_2 gas into the liquid phase to improve process productivity. Bioreactor configurations such as continuously stirred tank reactors (CSTR), hollow fiber reactors (HFR), and trickle bed reactors (TBR) are being considered to increase the gas-liquid mass transfer during syngas fermentation.

2.6 Reactors used in syngas fermentation

Various bioreactor designs have been developed and used to improve mass transfer between the liquid and gas phases. Bioreactors that achieve high mass transfer rates and high cell densities are most suitable for syngas fermentation. Gas/liquid mass transfer rates can be increased using high gas and liquid flow rates, high agitation rates (high specific gas-liquid interfacial areas) and increased gas solubility. The solubility of

gases can be enhanced by increasing the pressure inside the reactor or by addition of solvents and solvent mixtures such as 1-octene in water, acetonitrile, and phenol (Henstra et al. 2007; Purwanto et al. 1996; Xuan et al. 2008).

One of the reactors that is commonly used in syngas fermentation is CSTR because it can increase gas-to-liquid mass transfer rates. The CSTR is operated at high impeller speeds to enhance the overall mass transfer coefficient (K_La). High impeller speeds break up large bubbles into small bubbles, increasing the specific gas-liquid interfacial area (a) (Bredwell et al. 1999). Low rise velocities of small bubbles also increase the contact time between the gas bubble and liquid phase. The following design equation expresses K_La as a function of agitator power-per-volume ratio (P/V) and the superficial gas velocity (u_g).

$$K_La = K\left(\frac{P}{V}\right)^{\alpha}\left(u_g\right)^{\beta} \tag{2.29}$$

Where, K_L is the mass transfer coefficient, a is the gas/liquid interface area per liquid volume, α and β are constants that depend on reactor geometry, impeller type and design, and continuous phase properties. The increase in impeller speed would increase the power requirement for bioreactor operation. Under mass transfer limitations, increasing the impeller speed would increase (P/V), which further improves K_La (Equation 2.29). The increase in K_La enhances the reactor productivity. However, high P/V ratios would not make the process economical on an industrial scale. Moreover, increasing gas flow rates would also increase superficial gas velocity (u_g) and hence K_La. However for reactions involving sparingly soluble gases in the fermentation medium, high gas flow rates could result in low gas conversion efficiencies. Gas recycle can improve gas conversion efficiencies (Bredwell et al. 1999). In addition, the use of microspargers

improved the mass transfer coefficient several fold compared to conventional spargers. Moreover, the use of a multi-orifice ring sparger in a draft tube anaerobic bioreactor with a conical bottom surface increased gas holdup distribution as well as reduced poor mixing zones in the reactor (Varma and Al-Dahhan 2007).

CSTR and other reactor configurations such as packed-bubble column reactor (PBCR), and trickle bed reactor (TBR) were used in the fermentation of syngas to methane using a triculture *Rhodospirillum rubrum*, *Methanobacteriaum formicicum*, and *Methanosarcina barkeri* (Klasson et al. 1992b). Results demonstrated that increasing the agitation rate by 50% in the CSTR increased the mass transfer coefficient by 360%. The increase in agitation rates increased the power to volume ratio, which is not economically feasible for industrial scale reactors. The mass transfer rates were 26 times higher in TBR compared to PBCR. The low mass transfer rates in PBCR can be due to poor gas-liquid contact (Klasson et al. 1992b).

Also, the consumption of CO with respect to gas loading rates was compared for CSTR, PBCR, and TBR. It was found that the TBR had higher CO conversion and productivity at a given gas loading rate compared to CSTR and PBCR. However, CO conversion rates were higher in CSTR compared to PBCR (Klasson et al. 1992b).

The disadvantages of the TBR include plugging of the packed bed material due to microbial growth during the course of fermentation (Bredwell et al. 1999). Also controlling the pH of the medium is difficult in the TBR. However, this can be reduced by connecting a CSTR in series to the TBR. The fermentation broth can be mixed and pH can be adjusted in the CSTR. Therefore, it can be understood that pneumatically agitated reactors can be used to increase mass transfer rates. Also, pneumatically agitated

bioreactors provided good mass transfer with less power-to-volume ratio compared to CSTR (Bredwell et al. 1999).

Another major challenge in syngas fermentations beside mass transfer limitation due to low gas solubility is low cell yields (Vega et al. 1990; Worden et al. 1997). In order to get high product yields and productivity it is necessary to achieve high cell concentrations in the bioreactor. The use of cell immobilization techniques or cell recycle systems can improve cell densities in a reactor (Tsai et al. 2009b). Coskata Inc, claimed that microporous or non porous hollow fiber membrane reactors (HFR) have demonstrated dissolution and gas utilization rates as high as 100%, making them highly efficient and economical compared to other configurations (Tsai et al. 2009a). Another factor to make syngas fermentation potentially more economical is the use of inexpensive nutrients in the fermentation medium, which will be discussed in the next section.

2.7 Inexpensive nutrients sources for fermentation media

The production cost of ethanol is highly affected by the price of feedstocks, which accounts for about 60-75% of the total fuel cost (Balat and Balat 2009). In order to produce sustainable ethanol, it is necessary to optimize the fermentation process. One important consideration would be optimizing the fermentation medium. Nutrients in the fermentation medium play an important role in cell growth and product formation. Optimization of fermentation medium would eliminate components that are not required by the microorganism; and hence, reduce the overall cost of ethanol production. Inexpensive nutrients such as hydrolyzed soy flour, ethanol stillage, molasses, corn steep liquor (CSL) and other corn by-products are used for growing microorganisms in a

variety of industrial fermentations. The cost of these nutrients is less than $0.50 per kg (Witjitra et al. 1996).

Corn has been used as a carbohydrate source for many years in the acetone-butanol- ethanol fermentation by saccharolytic *Clostridia*. Corn mash has been used in batch fermentation for the production of solvents by *Clostridium acetobutylicum* P 262 (Guvenilir and Deveci 1996). Extruded corn broth was used for the production of butanol by a mutant strain of *Clostridium acetobutylicum* (Eden and Fuchs 1983).

CSL was also used as a nutrient in several fermentations. CSL is a byproduct of the initial stages of corn wet milling. It contains soluble components leached out from corn during soaking. It is rich in ammonia and amino acids (Witjitra et al. 1996). It also consists of vitamins, trace metals such as iron, manganese, boron, copper and zinc, and also contains lactic acid produced during steeping process by lactic acid producing bacteria. Also, CSL is rich in minerals, reducing sugars such as dextrose, organic acids (as phytic acids) and other nitrogen compounds. The composition of CSL slightly varies between manufacturers due to the source of corn used and various operating conditions.

CSL was used as an essential nutrient for growing various microorganisms and for solvent production by *Saccharomyces cerevisiae* (Kadam and Newman 1997), *Zymomonas mobilis* (Lawford and Rousseau 1997), and *C. beijerinckii* (Parekh et al. 1999). The lactic acid present in CSL was converted to acetic acid by *Clostridium thermoaceticum*; and hence, improved the yield of acetic acid (Shah and Cheryan 1995).

Another common nutrient used in fermentations is yeast extract, however, it is an expensive nutrient. Yeast extract is the major nutrient in the growth medium (ATCC medium no. 1754) designed for acetogenic *Clostridium* species. The cost of Bacto-yeast

extract from Difco Laboratories, Detroit, MI is, $157.49/kg (Racine and Saha 2007) and the cost of CSL on industrial scale is $0.07/kg (Lawford and Rousseau 1997; Wagner et al. 1983). The commercial cost of less expensive yeast extract, such as bacteriological yeast extract, Gistex yeast extract, and Expressa yeast extract are $22.03/kg, $8.81/kg and $8.81/kg, respectively. However, it has been found that these low cost yeast extracts did not perform well compared to Bacto-yeast extract ($157.49/kg) in the production of mannitol by *Lactobacillus intermedius* NNRL B-3693 (Saha 1996). Therefore, replacing yeast extract with CSL in industrial scale fermentation will significantly reduce fermentation costs. The low cost of CSL and its favorable composition encouraged our research team at Oklahoma State University to explore this inexpensive medium for syngas fermentations with *Clostridium* strain P11 to produce ethanol and other products.

The primary objective of this study was to compare the growth and product kinetics of strain P11 during syngas fermentation in both yeast extract and corn steep liquor media. Fermentation experiments were conducted in pressurized 250 mL serum bottles and in a 7.5 L bench scale fermentor. The effect of both media components on the rate of product formation and syngas conversion efficiencies was determined. This work evaluated and validated the incorporation of CSL as an inexpensive raw material into the fermentation medium for ethanol production through syngas fermentation by P11.

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CHAPTER III

EFFECT OF CORN STEEP LIQUOR AND YEAST EXTRACT ON SYNGAS FERMENTATION BY *CLOSTRIDIUM* STRAIN P11 IN 250-mL SERUM BOTTLES

3.1 Introduction

Gasification-fermentation is a novel technology for the production of biofuels from a variety of organic matter such as dedicated crops, agricultural residues, municipal solid wastes, forestry wastes, grasses and other biological wastes. Gasification is a thermochemical process that converts all non mineral components in the biomass including lignin to yield synthesis or producer gas, which is primarily composed of CO, CO_2 and H_2. Anaerobic microorganisms, such as acetogens and methanogens, could serve as catalysts for the conversion of inorganic substrates such as CO, CO_2 and H_2 into fuel (Najafpour and Younesi 2006). Acetogens are capable of metabolizing single-carbon compounds to produce ethanol and other high molecular weight products via acetogenic fermentation (Worden et al. 1991). Synthesis gas (syngas) can be metabolized to ethanol and butanol by several microbial catalysts such as *Bacillus methylotrophicum* and *Clostridium ljungdahlii* (Vega et al. 1989; Worden et al. 1991). A few *Clostridium* species, such as *Clostridium ljungdahlii* (Phillips et al. 1994) *Clostridium autoethanogenum* (Abrini et al. 1994), *Clostridium carboxidivorans*

(Rajagopalan et al. 2002), *Clostridium* strain P11 (Frankman 2009; Panneerselvam 2009; Saxena 2008) can grow on syngas components and produce ethanol and acetic acid. These microorganisms utilize the reductive acetyl-CoA pathway, also known as the "Wood-Ljungdahl" pathway, for the synthesis of acetyl-CoA, for conservation of energy and growth. The acetyl-CoA formed is further reduced to ethanol and other products (Drake 1992).

Clostridium strain P11 has the ability to grow autotrophically on gas mixtures containing CO/CO_2 or H_2/CO_2. Also it can grow chemoorganotrophically on substrates such as glucose and fructose to produce ethanol and acetate (Huhnke et al. 2008; Panneerselvam 2009; Saxena 2008). Acetogenic bacteria utilize acetyl-CoA pathway for growth on inorganic substrates such as CO and CO_2 and production of reduced products such as ethanol (Wood et al. 1986). The stoichiometry and Gibbs free energies (ΔG^o) for ethanol and acetic acid production from different gas components are shown in the Table 3.1.

Gibbs free energy indicates whether a reaction is spontaneous. A reaction is said to be spontaneous if the Gibbs free energy is negative. Ethanol is produced from both CO and H_2 through Equations 3.1 and 3.2. Ethanol production from CO as a substrate (Equation 3.1) is more thermodynamically favorable than from H_2 at standard conditions (Equitation 3.2).

The equations in Table 3.1 do not account for carbon assimilated into cell mass. If CO is the sole source of carbon and energy, then one-third of carbon from CO theoretically can be converted to ethanol and the remaining two-thirds is converted to CO_2 (Equation 3.1).

Table 3.1 Stoichiometries and Gibbs free energy for ethanol and acetic acid production from gases present in syngas (Phillips et al. 1994) .

	ΔG^o (kJ/mol)	Equation
$6CO + 3H_2O \rightarrow C_2H_5OH + 4CO_2$	-225	3.1
$6H_2 + 2CO_2 \rightarrow C_2H_5OH + 3H_2O$	-105	3.2
$4CO + 2H_2O \rightarrow CH_3COOH + 2CO_2$	-175	3.3
$2CO_2 + 4H_2 \rightarrow CH_3COOH + 2H_2O$	-95	3.4

When equal moles of CO and H_2 are supplied, theoretically two-thirds of the carbon from CO can be converted to ethanol and the remaining carbon is accounted for CO_2 production as can be seen by combining Equations 3.1 and 3.2.

$$3CO + 3H_2 \rightarrow C_2H_5OH + CO_2 \quad (3.5)$$

Acetic acid is also produced from CO and H_2. Combining Equations 3.3 and 3.4 shows that all the carbon supplied in the form of CO theoretically can be converted to acetic acid.

$$2CO + 2H_2 \rightarrow CH_3COOH \quad (3.6)$$

Butanol is another product that can be produced from syngas as follows:

$$12CO + 5H_2O \rightarrow C_4H_9OH + 8CO_2 \quad (3.7)$$

$$12H_2 + 4CO_2 \rightarrow C_4H_9OH + 7H_2O \quad (3.8)$$

Microorganisms have different nutrient requirements for growth and product formation. Standard *Clostridium* strain P11 medium is composed of yeast extract, vitamins, minerals, trace metals and reducing agent (Saxena 2008). These components are expensive, which increase the cost of ethanol production when used on an industrial scale. Using inexpensive nutrient sources rich in vitamins, minerals and carbohydrates would be more desirable. Some inexpensive nutrients that could replace yeast extract include corn steep liquor (CSL), hydrolyzed cotton seed flour, hydrolyzed soy flour and ethanol stillage (Witjitra et al. 1996).

CSL is a major by-product from the corn wet milling industry. It is a rich source of vitamins, minerals, amino acids and proteins (Azeredo et al. 2006; Kadam and Newman 1997). It has been used as growth medium for mannitol production by *Lactobacillus intermedius* (Saha 1996), antibiotic production (El-Marsafy et al. 1975; Yang and Lee 2001), enzyme production (Grigorevski de Lima et al. 2005), and solvent production by *Saccharomyces cerevisiae* (Kadam and Newman 1997), *Clostridium beijerinckii* (Parekh et al. 1999) and *Zymomonas mobilis* (Lawford and Rousseau 1997). CSL is selected in this present study as an alternative to yeast extract for syngas fermentation because it is rich in nutrients and inexpensive. The cost of Bacto-yeast extract from Difco Laboratories (Detroit, MI) is $157.49/kg (Racine and Saha 2007), while the cost of CSL on an industrial scale is $0.07/kg (Lawford and Rousseau 1997; Wagner et al. 1983).

The objective of this study was to evaluate corn steep liquor as an alternate nutrient to yeast extract for syngas fermentation using *Clostridium* strain P11.

3.2 Materials and methods

3.2.1 Microorganism

A novel *Clostridium* strain P11 (ATCC PTA-7826) provided by Dr. Ralph Tanner, University of Oklahoma, was used. Strain P11 is a gram positive, rarely motile, rod shaped bacterium and occurs singly or in chains. It has the ability to grow autotrophically on gas mixtures containing CO/CO_2 or H_2/CO_2. Also, it can grow chemoorganotrophically to produce ethanol and acetate. The optimum temperature and pH for this microorganism were found to be 37°C and 6.10, respectively (Saxena 2008).

In order to reduce the lag phase and also to ensure that viable cells are inoculated into the medium, cells from the stock inoculum were passaged two times prior to inoculation. Cell passaging and syngas fermentation were performed in 250 mL serum bottles with 100 mL working volume. The first passage was inoculated with 10% (v/v) of inoculum and then purged with syngas at 239 kPa (absolute). When the OD in the first passage reached about 0.5 units when measured at 660 nm, cells were transferred to the second passage. Finally, when the OD reached 0.5 units in the second passage, 10% (v/v) of cells were transferred to the test bottles to follow syngas fermentation.

3.2.2 Fermentation medium

Strain P11 is highly sensitive to oxygen; and hence, the fermentation was performed under strict anoxic conditions. The fermentation medium contained minerals, trace metals, vitamins and reducing agents (Saxena 2008). The mineral stock solution contained (per liter) 100 g ammonium chloride, 4 g calcium chloride, 20 g magnesium sulfate, 10 g potassium chloride and 10 g potassium phosphate monobasic. The trace metal composition (per liter) was 0.2 g cobalt chloride, 0.8 g ferrous ammonium sulfate,

1 g manganese sulfate, 0.2 g nickel chloride, 2 g nitrilotriacetic acid, 0.02 g sodium molybdate, 0.1 g sodium selenate, 0.2 g sodium tungstate and 1 g zinc sulfate. The stock vitamin solution (per liter) contained 0.005 g of p-amino benzoic acid, 0.002 g d-biotin, 0.005 g pantothenic acid, 0.002 g folic acid, 0.01 g 2-MercaptoEthane Sulfonate Sodium (MESNA), 0.005 g nicotinic acid, 0.01 g pyridoxine, 0.005 g riboflavin, 0.005 g thiamine, 0.005 g thioctic acid and 0.005 g vitamin B-12. Unless mentioned, all media components were purchased from Sigma Aldrich (St. Louis, MO). Yeast extract (Difco laboratories, Detroit, MI) and corn steep liquor (Sigma Aldrich, St. Louis, MO) were used as complex nitrogen and nutrient sources in the fermentation medium.

In order to produce consistent results, CSL from the same batch was used throughout the study. CSL contains about 50% solids. Before the addition of CSL into the fermentation medium, the solids from crude CSL were removed by centrifugation at 13000 rpm for 10 min. Resazurin solution (0.1%) was added into the medium as a redox indicator. Morpholinoethanesulfonic acid (MES) was added as a biological buffer to prevent excessive fluctuations in the pH during fermentation. The initial pH of the medium was adjusted to 6.05 before inoculation, using 2 N KOH. Three different fermentation media optimized by Saxena (2008) were used in the present study (Table 3.2). The analysis of sugar content of the liquid portion of the crude CSL was done using high pressure liquid chromatography (HPLC) **(**Agilent 1100 series, Wilmington, DE**)** with refractive index detector. The column used was Aminex HPX 87P (Bio-Rad, Sunnyvale, CA, USA), which was operated at 85°C with de-ionized water as the mobile phase pumped at 0.6 mL/min for 30 min per sample. The liquid portion of the crude CSL

contained 33 g/L cellobiose, 11 g/L glucose, 5 g/L xylose, 6 g/L galactose, 7 g/L arabinose and 3 g/L mannose. The total sugar content in the crude CSL is 65 g/L.

Table 3.2 Composition of various media used for syngas fermentation.

	Standard-Yeast extract 1 g/L	CSL 10 g/L	CSL 20 g/L
Yeast extract (g)	1	-	-
Corn steep liquor (g)	-	10	20
Minerals (mL)	30	30	-
Trace metals (mL)	10	10	10
Vitamins (mL)	10	10	-
MES (g)	10	10	10
0.1% Resazurin (mL)	1	1	1
Ammonium chloride (g)	-	-	1.25
4% Cysteine sulfide (mL)	10	10	2.5
Total volume, (L)	1	1	1

3.2.3 Syngas

Commercial syngas with gas composition similar to producer gas generated from our gasification facility was used. The syngas was composed of 20% CO, 15% CO_2, 5% H_2 and 60% N_2 (volume %).

3.2.4 Batch studies

Batch fermentations were conducted in 250 mL serum bottles (Wheaton, NJ) with 100 mL working volume containing media provided in Table 3.2. All media components except reducing agent were well mixed in deionized water and pH was adjusted to 6.05

using 2N KOH. The media were boiled and then purged with nitrogen for 3 minutes to remove oxygen. The serum bottles were sealed using gas impermeable butyl rubber septum-type stoppers and aluminum crimp seals. One mL of 4% cysteine sulfide solution was added to all media to scavenge any remaining dissolved oxygen. The fermentation media were autoclaved (Primus Sterilizer Co. Inc, Omaha, NE) at 121°C for 20 min.

Before inoculating the new medium, the seed culture was pre-warmed to 37°C. Media were inoculated with 10% (v/v) of strain P11 cluture. Sterile 0.2 µm PTFE (polytetrafluoroethylene) membrane filters (VWR International, West Chester, PA) were used in the syngas inlet line that fed to strain P11 at 239 kPa (absolute). The bottles were placed vertically on an orbital shaker (Innova 2100, New Brunswick Scientific, Edison, NJ) with agitation speed of 150 rpm and 37°C.

All studies were performed in triplicate with fermentation carried out for over 600 h. The pressure inside the bottle was measured before taking liquid and gas samples every 24 h. Gas samples were withdrawn in anaerobic glove box (Coy Laboratory Products Inc., Grasslake, MI) using 100 µL gas tight sample lock syringes (Hamilton Company, Reno, Nevada). Liquid samples of 2.0 mL were withdrawn under aseptic conditions in a biosafety cabinet using a sterile syringe; 0.5 mL of the liquid sample was used for measuring optical density at 660 nm, and pH was measured using the remaining sample. After measuring pH, the sample was centrifuged at 13000 rpm for 13 min. The supernatant was filtered using 0.45 µm nylon membrane filters (VWR International, West Chester, PA) and frozen for solvent analysis. The gas in the head space in each bottle was replaced every 24h with fresh syngas at 239 kPa (absolute).

3.2.5 Analytical procedures

3.2.5.1 Cell concentration

Cell concentration was determined using a UV-Vis spectrophotometer (Varian Inc., Palo Alto, CA). Liquid samples were collected in 2 mL cuvettes from the serum bottles and optical density (OD) was measured at 660 nm. Samples with OD values above 0.4 unit, were diluted so that the OD was within the linear range of the calibration curve. One unit of OD corresponds to 0.34 g/L cell mass (Panneerselvam 2009).

3.2.5.2 Solvent analysis

Liquid samples were centrifuged at 13,000 rpm for 13 min. The supernatant was filtered through 0.45 µm nylon membrane filters (VWR International, West Chester, PA) and frozen until further analysis. Ethanol, acetic acid and butanol concentrations were analyzed using 6890 Gas Chromatography (GC), (Agilent Technologies, Wilmington, DE). A PoraPak QS 80/100 **(**Alltech, Deerfield, IL) packed column connected to a flame ionization detector (FID) was used. The GC was operated under isothermal conditions with the oven temperature set at 210°C. Helium was used as a carrier gas with a flow rate of 25 mL/min. The resulting chromatograms were analyzed using CHEMSTATION® data analysis package. The percentages of error in ethanol, acetic acid and butanol measurements were less than 5%.

3.2.5.3 Gas analysis

A GC equipped with thermal conductivity detector (TCD) was used for gas analysis. A capillary column, Carboxen 1010 PLOT (Supelco, Bellefonte, PA), was used to detect and quantify CO, CO_2, H_2 and N_2. The gas analysis was conducted in duplicate for each experiment. Argon was used as a carrier gas in the GC with an initial gas flow

rate of 0.4 mL/min for the first 12 min, and then it was increased at a rate of 0.1 mL/min until reaching 0.8 mL/min. The oven temperature was set at 32°C for 12 min, after which the temperature was increased at a rate of 30°C per min until it reached 236°C. The temperatures of the column inlet and detector were set at 200°C and 230°C, respectively. Resulting chromatograms were analyzed using CHEMSTATION® data analysis package. The GC was calibrated by injecting samples with different volumes of known gas concentrations. The calibration standards were frequently injected to check the accuracy of GC. The percentages of error in CO, CO_2, H_2 and N_2 measurements were below 5%.

3.2.5.4 Statistical Analysis

An analysis of variance (ANOVA) was determined using the GLM procedure of SAS Release 9.2 (Cary, NC, USA). A Dunnett's test (SAS Institute, Cary, NC) was used to determine the statistical significance of the fermentation parameters (cell mass, ethanol, butanol and acetic acid) between the different media used. Significance level was tested at $p = 0.05$.

3.3 Results and discussion

3.3.1 Fermentation pattern

The growth and product profiles of strain P11 in 1 g/L yeast extract and 10 g/L and 20 g/L CSL media are shown in Figures 3.1 to 3.3.

3.3.1.1 Yeast extract medium

The cell concentration in 1 g/L yeast extract (YE) medium increased exponentially from 0.04 g/L to 0.23 g/L during the first 48 h with a specific growth rate of 0.059 h^{-1} (Figure 3.1). Cell concentration was stable at 0.22 g/L until 288h, after which it slightly decreased to 0.19 g/L at 600 h. A maximum cell concentration of 0.25 g/L was

obtained at 120 h. The pH decreased from 6.05 to 4.81 within the first 168 h. This decrease in pH was due to acetic acid production during cell growth. Subsequently, an increase in pH to 5.11 and a decrease in the concentration of acetic acid was observed between 168 and 360 h. A slight drop in pH from 5.11 to 4.88 was noted between 360 and 600 h due to slight increase in acetic acid production.

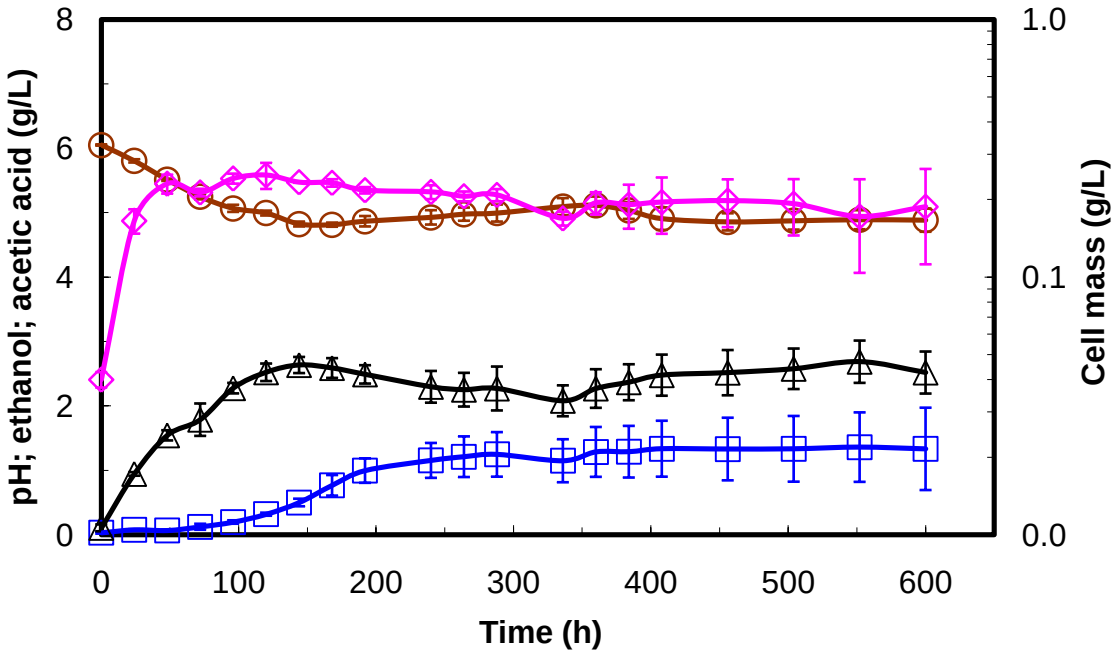

Figure 3.1 Growth, pH and product profiles using *Clostridium* strain P11 in 1 g/L yeast extract medium: (◇) cell mass; (□) ethanol; (Δ) acetic acid; (O) pH.

Strain P11 produced ethanol and acetic acid as the major fermentation products. Acetic acid was produced during growth phase of strain P11 and reached a maximum of 2.63 g/L after 144 h. Acetic acid concentration then decreased to 2.07 g/L between 144 and 336 h, followed by an increase to 2.68 g/L at 552 h. The increase in acetic acid after 336 h was due to growth resumption. The maximum acetic acid concentration was 2.68 g/L at 552 h with a corresponding productivity of 4.2 mg/Lh.

Ethanol production started in the stationary phase after 72 h of fermentation. However, most of the ethanol production occurred between 96 and 192 h. A maximum ethanol concentration of 1.36 g/L was obtained after 552 h with a corresponding productivity of 2.2 mg/Lh. A decrease in acetic acid concentration between 120 and 336 h corresponded with an increase in ethanol production. This suggests that acetic acid was converted to ethanol by strain P11. This was also observed in other syngas fermentation studies using *Clostridium* strain P11 (Frankman 2009; Panneerselvam 2009; Saxena 2008) and *Clostridium carboxidivorans* $P7^T$ (Hurst and Lewis 2010). Some butanol production was detected in the 1 g/L YE medium in the current study with strain P11. About 60 mg/L of butanol was produced after 600 h of fermentation.

3.3.1.2 CSL medium

Similar to yeast extract medium, no lag phase was observed in either the 10 g/L or 20 g/L CSL media (Figures 3.2 and 3.3). The cell concentration increased exponentially from 0.06 g/L to 0.23 g/L in the medium with 10 g/L CSL during the first 48 h (Figure 3.2). The specific growth rate was 0.049 h^{-1} which is 20% lower than in yeast extract medium. The cell concentration was constant at 0.3 g/L until 264 h, followed by an increase to 0.42 g/L till 600 h. There were 68% more cells produced in 10 g/L CSL medium compared to YE medium. In 10 g/L CSL medium, a decrease in pH from 6.04 to 5.11 was observed during growth with simultaneous production of acetic acid during the fermentation. The pH was stable around 5.1 from 240 to 600 h. Statistical analysis showed that the pH in 20 g/L CSL medium was significantly higher compared to 1 g/L YE and 10 g/L CSL media throughout the fermentation time ($p < 0.05$). Also, the

pH in 10 g/L CSL medium was significantly higher compared to 1 g/L YE medium between 72 h to 264 h ($p < 0.05$).

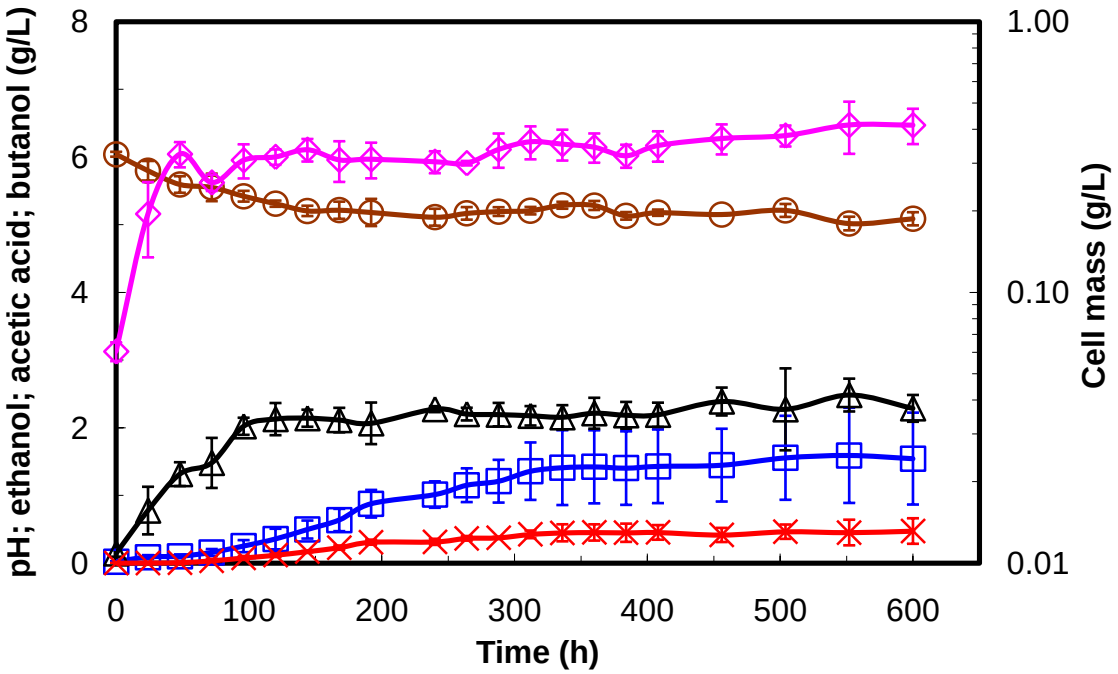

Figure 3.2 Growth, pH and product profiles using *Clostridium* strain P11 in 10 g/L corn steep liquor medium: (◇) cell mass; (□) ethanol; (Δ) acetic acid; (O) pH; (×) Butanol.

In the 20 g/L CSL medium, the cell concentration increased exponentially from 0.07 g/L to 0.42 g/L within the first 24 h of cultivation (Figure 3.3). The specific growth rate of P11 in this medium was 0.076 h^{-1} which is 29% and 55% higher than in YE and 10 g/L CSL media, respectively. The cell concentration in the 20 g/L CSL medium was constant at 0.38 g/L from 24 to 216 h followed by a death phase. Statistical analysis indicated that the cell mass in 10 g/L and 20 g/L CSL media between 24 h and 600 h was significantly higher than standard yeast extract medium, confirming that nutrients present in CSL are significantly enhancing cell growth ($p < 0.05$). Also, cell mass in 10 g/L CSL between 24 h and 264 h was significantly less compared to 20 g/L CSL medium.

However, there was no significant difference in cell mass after 264 h of fermentation within the CSL media ($p < 0.05$).

During cell growth, the pH of the medium decreased from 5.90 to 5.38 with acetic acid as the primary product. An increase in the pH of the medium was observed after 48 h with the onset of ethanol and butanol production and it continued to increase to 6.0 after 600 h of fermentation. The increase in the pH was accompanied by a decrease in acetic acid concentration (Figure 3.3).

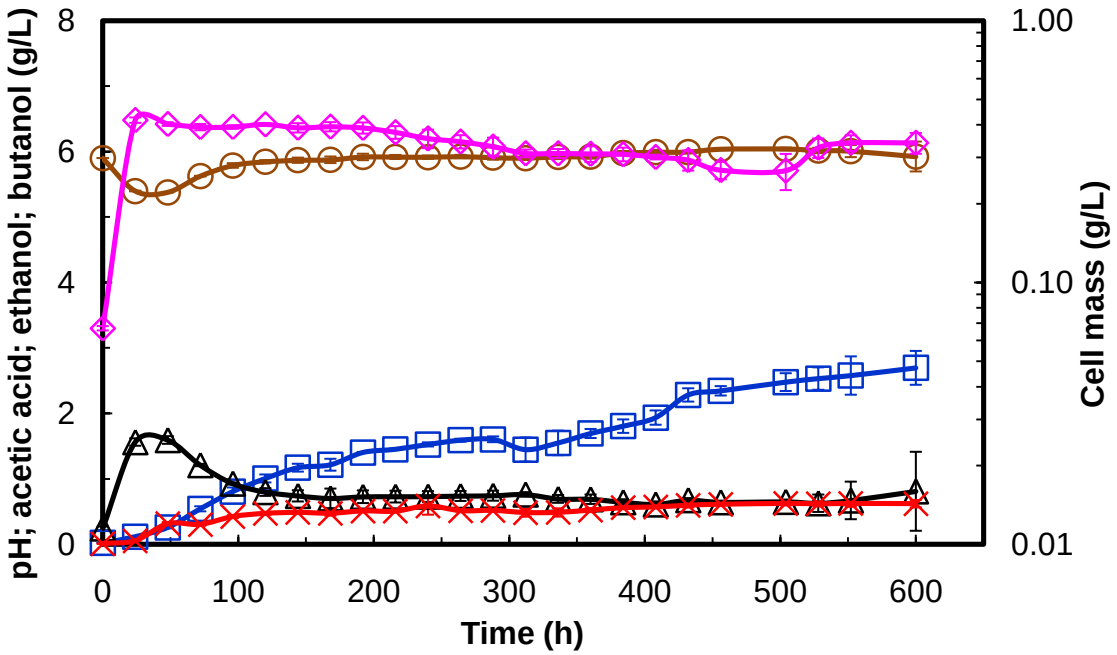

Figure 3.3 Growth, pH and product profiles using *Clostridium* strain P11 in 20 g/L corn steep liquor medium: (◇) cell mass; (□) ethanol; (Δ) acetic acid; (O) pH; (×) Butanol.

Product profiles of strain P11 in 10 g/L and 20 g/L CSL media are shown in Figures 3.2 and 3.3, respectively. Acetic acid was produced during growth phase. A maximum acetic acid concentration of 2.48 g/L was obtained after 552 h with a corresponding productivity of 3.8 mg/Lh in the 10 g/L CSL medium. However, the maximum acetic acid concentration was 1.59 g/L after 48 h with a corresponding

productivity of 1.3 mg/Lh in the 20 g/L CSL medium (Figure 3.3). There was a significant decrease in acetic acid concentration after 96 h of fermentation in the 20 g/L CSL medium compared to both 1 g/L YE and 10 g/L CSL media ($p < 0.05$). This further confirms that strain P11 in the 20 g/L CSL medium consumed acetic acid to probably produce ethanol. Moreover, there was no significant difference in acetic acid concentration between the 1 g/L YE and 10 g/L CSL media during fermentation ($p < 0.05$).

Ethanol production started after 72 h of incubation with a maximum concentration of 1.54 g/L after 600 h and a corresponding productivity of 2.5 mg/Lh in the 10 g/L CSL medium (Figure 3.2). Along with ethanol another higher alcohol, butanol, was produced with a maximum concentration of 0.47 g/L after 600 h. In the 20 g/L CSL medium, ethanol and butanol production started after 48 h of incubation and the production rate was at its maximum between 24 and 192 h (Figure 3.3). The maximum ethanol and butanol concentrations were 2.69 g/L and 0.62 g/L after 600 h, respectively. The ethanol productivity was 4.5 mg/Lh. The ethanol concentration in 20 g/L CSL medium was 108% and 80% higher than in 1 g/L YE and 10 g/L CSL media, respectively. Statistical analysis proved that the 20 g/L CSL medium significantly produced more ethanol from 48 h to 600 h of fermentation compared to the 1 g/L YE medium ($p < 0.05$). The amount of ethanol produced by strain P11 in the 20 g/L CSL medium (2.7 g/L) was significantly higher compared to the1 g/L YE medium (1.3 g/L). However, there was no significant difference in ethanol concentrations between 10 g/L CSL and 1 g/L YE media throughout the fermentation ($p < 0.05$). Although the amount of ethanol produced in 20 g/L CSL medium between 48 h and 240 h was significantly higher than in the 10 g/L CSL

medium, there was no significant difference in ethanol concentration at 600 h of fermentation between these two media ($p < 0.05$). The amount of butanol produced in 10 g/L and 20 g/L CSL media were significantly higher compared to the 1 g/L YE medium ($p < 0.05$). Moreover, there was no significant difference in the amount of butanol produced between 10 g/L CSL and 20 g/L CSL media ($p < 0.05$).

3.3.2 Gas consumption and pressure profiles

The syngas used in batch studies was composed of 20% CO, 15% CO_2, 5% H_2 and 60% N_2 (volume %). The CO and H_2 present in the syngas were utilized by strain P11 for growth and production of acetic acid, ethanol and butanol.

3.3.2.1 Yeast extract medium

CO and H_2 consumption, CO_2 production and pressure profiles during syngas fermentation using P11 in 1 g/L yeast extract medium are shown in Figure 3.4. The gas analysis was not completed beyond 336 h due to technical problems. A pressure reading was recorded every 24 h before collecting the liquid and gas samples for analysis. After taking liquid and gas samples, the bottles were re-pressurized to the initial value of 239 kPa (absolute) using fresh syngas. A decrease in the pressure inside the serum bottles indicated a consumption of syngas components. A decrease in the pressure was observed during cell growth and product formation. The pressure was not changed from the initial value of 239 kPa (absolute) after 264 h, which indicates no syngas consumption by P11.

Within the first 48 h, about 65% and 76% of the supplied CO and H_2, respectively, were consumed during growth and acetic acid production (Figure 3.4). This implies that both CO and H_2 were utilized by strain P11 for growth, acetic acid and CO_2 production. The production of CO_2 increased simultaneously with the increase in CO

consumption. In the stationary phase from 48 to 144 h, the CO and H_2 utilization remained constant at 65% and 75%, respectively. During this period, acetic acid and ethanol were produced simultaneously by the cells. However, more acetic acid was produced compared to ethanol during this period. In contrast, ethanol production was favored by the cell after 144 h, while acetic acid concentration slightly decreased.

Less H_2 consumption was noticed with ethanol formation, which indicates that CO was the main syngas component used to maintain P11 cellular activity to produce ethanol. CO and H_2 consumption decreased gradually to 4% and 13%, respectively, at 336 h. However, ethanol production continued at a very low rate for the remainder of the fermentation run.

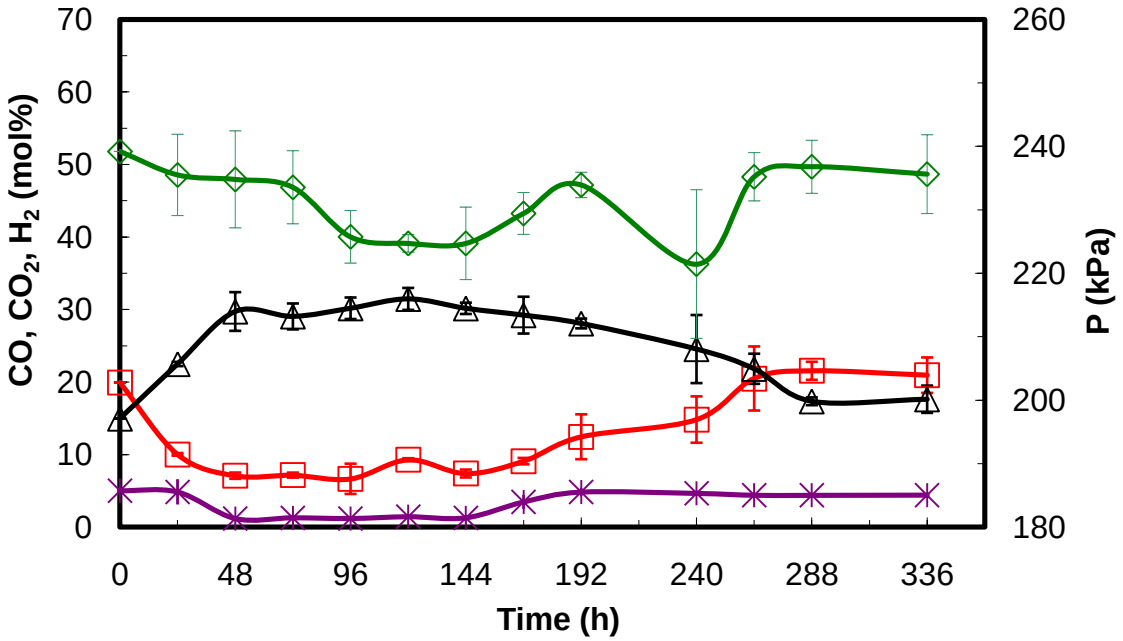

Figure 3.4 CO, CO_2, H_2 and pressure profiles using *Clostridium* strain P11 in 1 g/L yeast extract medium: (□) CO; (Δ) CO_2; (×) H_2; (◇) pressure.

The total number of moles of CO and H_2 consumed within 336 h of fermentation in YE medium were 17.72 and 4.23 mmol, respectively (Figure 3.5). About 91% of the

total moles of CO consumed in the first 336 h were utilized within the first 192 h of the fermentation. During this period, cell growth, acetic acid and ethanol production were noticed (Figure 3.1). H_2 consumption leveled off similar to the acetic acid profile. About 88% of the total H_2 consumed during 336 h was utilized within the first 192 h.

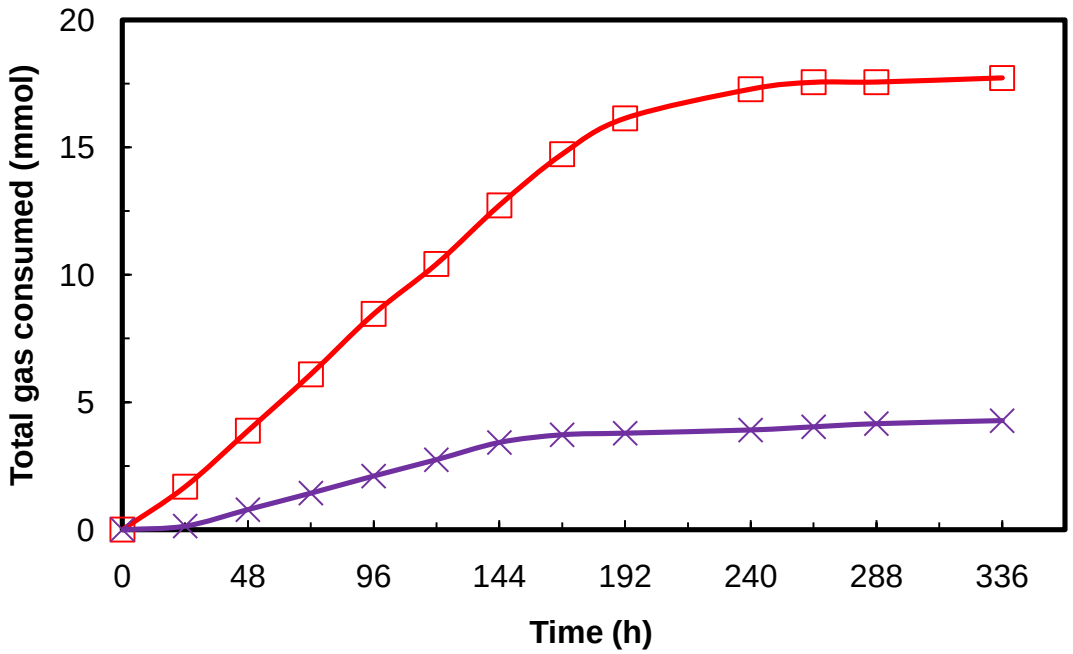

Figure 3.5 Total moles of CO and H_2 consumed by *Clostridium* strain P11 in 1 g/L yeast extract medium: (□) CO; (×) H_2.

The specific uptake rates (mmoles of substrate/min. g cells) of CO (q_{CO}) and H_2 (q_{H2}) are shown in Figure 3.6. CO and H_2 uptake rates increased sharply during the growth phase from 0 to 48 h and remained nearly constant from 48 to 144 h. Then, the specific uptake rate H_2 sharply decreased between 144 and 192 h due to decline in H_2 consumption. The CO specific uptake rate also decreased after 144 h due to low CO consumption by P11.

Ethanol yield was calculated based on CO consumption during syngas fermentation because insignificant amount of H_2 was consumed during ethanol

production. The conversion efficiencies of CO and H_2 at 336 h were 39% and 37%, respectively (Table 3.3). Sample calculation for the conversion efficiencies is shown in Appendix C. The ethanol and acetic acid yields based on CO consumption were 73% and 67% of the theoretical values, respectively.

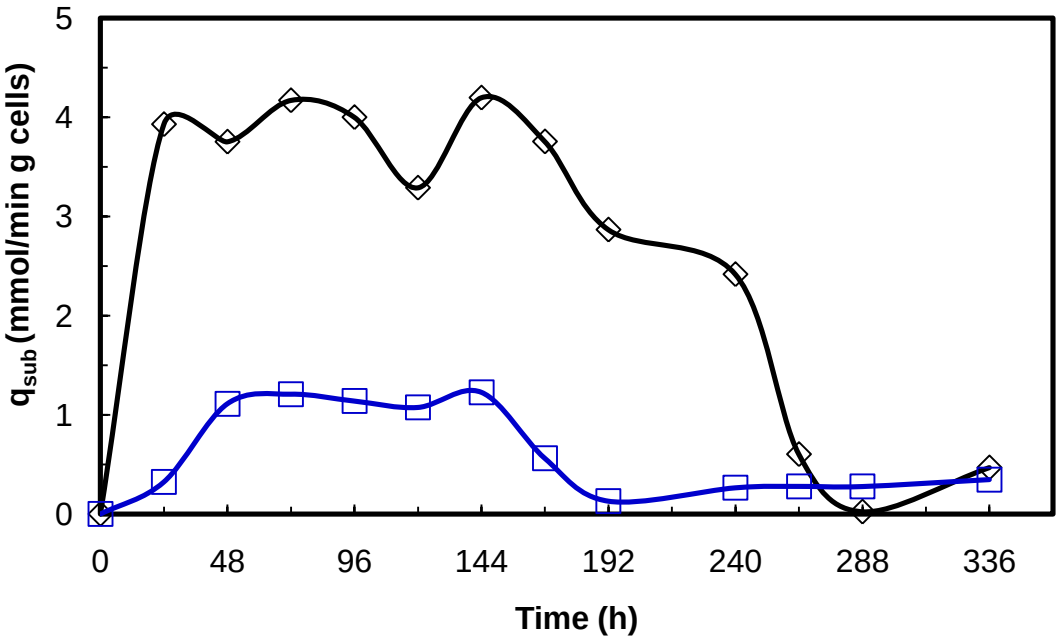

Figure 3.6 Specific uptake rates (q_{sub}) of CO and H_2 by *Clostridium* strain P11 in 1 g/L yeast extract medium: (◇) CO; (□) H_2.

Table 3.3 Fermentation characteristics for *Clostridium* strain P11 in yeast extract and corn steep liquor media.

	Medium		
	YE 1 g/L	CSL 10 g/L	CSL 20 g/L
μ_{max} (h^{-1})	0.059	0.049	0.076
Final pH [b]	4.96	5.09	5.92
Acetic acid[b] (g/L)	2.5	2.3	0.8
Ethanol[b] (g/L)	1.3	1.5	2.7
Butanol[b] (g/L)	0.1	0.5	0.6
Ethanol productivity[b] (mg/ Lh)	2.2	2.5	4.5
Acetic acid productivity[b] (mg/ Lh)	4.2	3.8	1.3
Ethanol yield from CO (% of theoretical value)	73[a]	79[a] / 60[b]	142[a] / 228[b]
Ethanol yield from CO (% of theoretical value)[c]	N/A	N/A	46[a] / 129[b]
Acetic acid yield from CO (% of theoretical value)	67[a]	65[a] / 45[b]	33[a] / 35[b]
Conversion efficiency of CO, (%)	39[a]	41[a] / 29[b]	23[a] / 14[b]
Conversion efficiency of H_2, (%)	37[a]	25[a] / 22[b]	1[a] / 1[a]

[a] Values calculated at 336 h
[b] Values calculated at 600 h
[c] Values calculated considering acetic acid conversion to ethanol
N/A=Not applicable, no net acetic acid consumption was noticed

3.3.2.2 CSL medium

Figures 3.7 and 3.8 depict CO and H_2 consumption, CO_2 production and pressure profiles during syngas fermentation using P11 in 10 g/L and 20 g/L CSL media, respectively. The pressure profile in the medium with 10 g/L CSL (Figure 3.7) was similar to YE medium (Figure 3.4) in which the pressure inside the serum bottles decreased for the first 240 h of fermentation. Then, less syngas was consumed during the remaining 360 h of fermentation. However, in the 20 g/L CSL medium, the change in pressure was measured only for a short period of time because the utilization of CO and H_2 decreased within the first 200 h of fermentation (Figure 3.8).

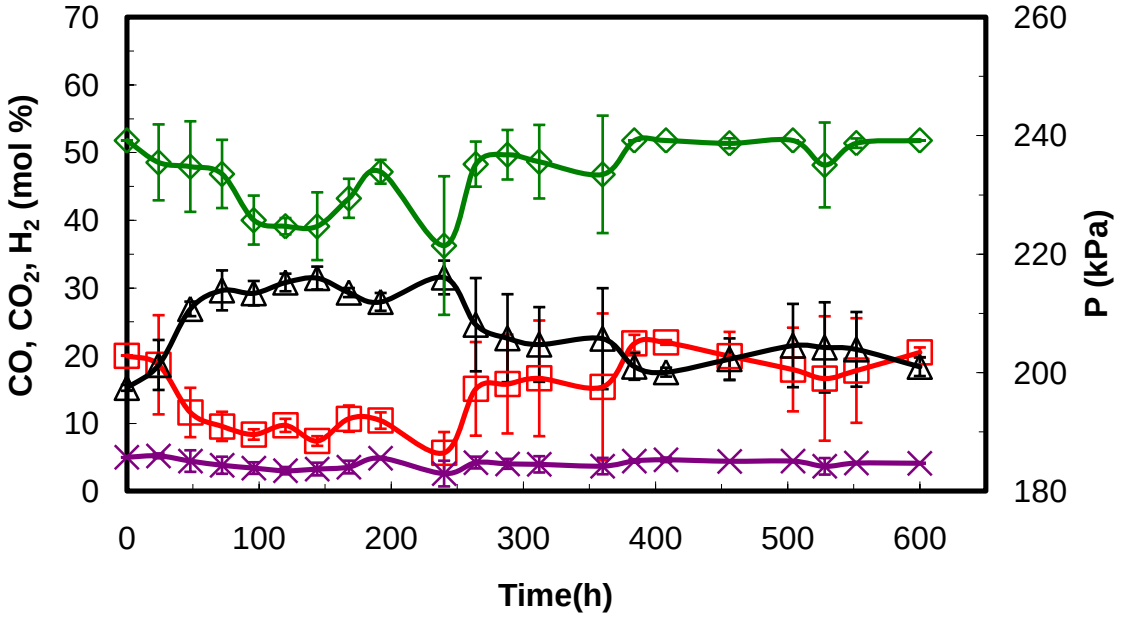

Figure 3.7 CO, CO_2, H_2 and pressure profiles using *Clostridium* strain P11 in 10 g/L corn steep liquor medium: (□) CO; (Δ) CO_2; (×) H_2; (◇) pressure.

A decrease in pressure was observed during cell growth and product formation due to CO and slight H_2 utilization by strain P11. The pressure in the bottles with 10 g/L CSL did not change from the initial value of 239 kPa after 384 h due to reduced syngas

utilization. The amount of CO consumed increased after 24 h of fermentation. The maximum percentage of CO consumption was 65% at 144 h. However, the maximum H_2 consumption (45%) was observed at 120 h. During this period, mainly cell growth and acetic acid production occurred. Most of the CO consumed after 96 h was accounted for in cell maintenance and ethanol production. After 240 h, the percentage of CO and H_2 consumption decreased and remained steady at about 25%. However, ethanol production continued at a slow rate for the rest of the fermentation.

In the 20 g/L CSL medium, maximum consumption of CO of 71% was measured after 72 h (Figure 3.8). No CO utilization by P11 was measured after 192 h. Subsequent decrease in CO_2 production was also observed after 192 h. Moreover, H_2 consumption was observed only during the exponential growth phase (0 to 24 h), which indicates that H_2 was mainly utilized for the production of acetic acid and most of the CO consumption was directed to ethanol and butanol formation.

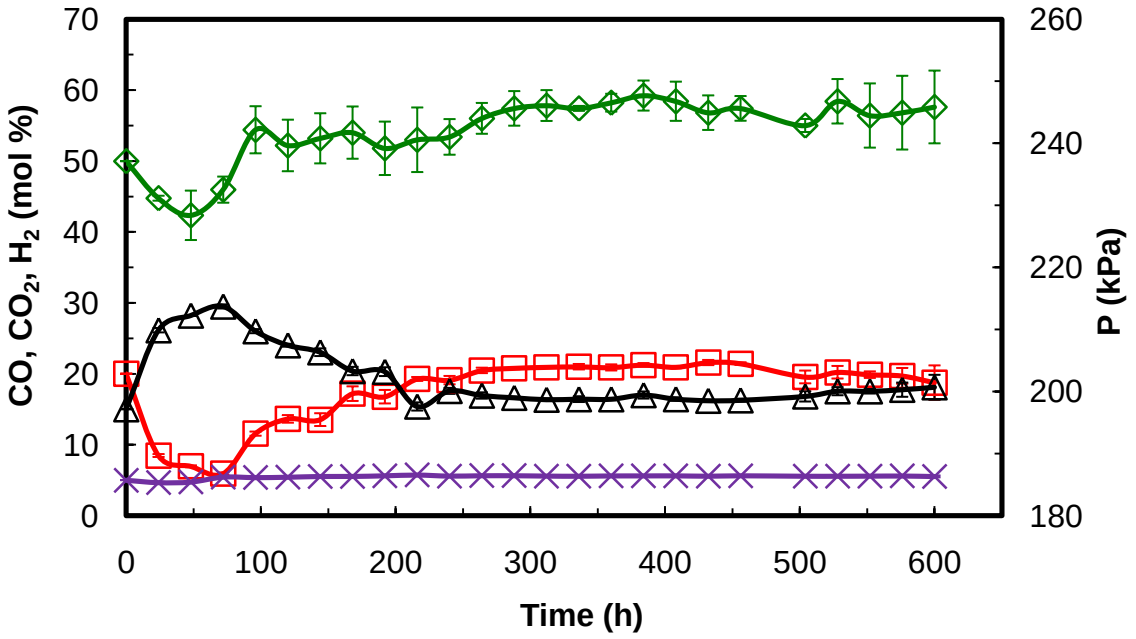

Figure 3.8 CO, CO_2, H_2 and pressure profiles using *Clostridium* strain P11 in 20 g/L corn steep liquor medium: (□) CO; (Δ) CO_2; (×) H_2; (◇) pressure.

The total number of moles of CO and H_2 consumed during syngas fermentation in 10 g/L CSL medium were 22.74 and 4.33 mmol, respectively (Figure 3.9); whereas, only 12.47 and 0.16 mmol of CO and H_2, respectively, were consumed in the 20 g/L CSL medium (Figure 3.10).

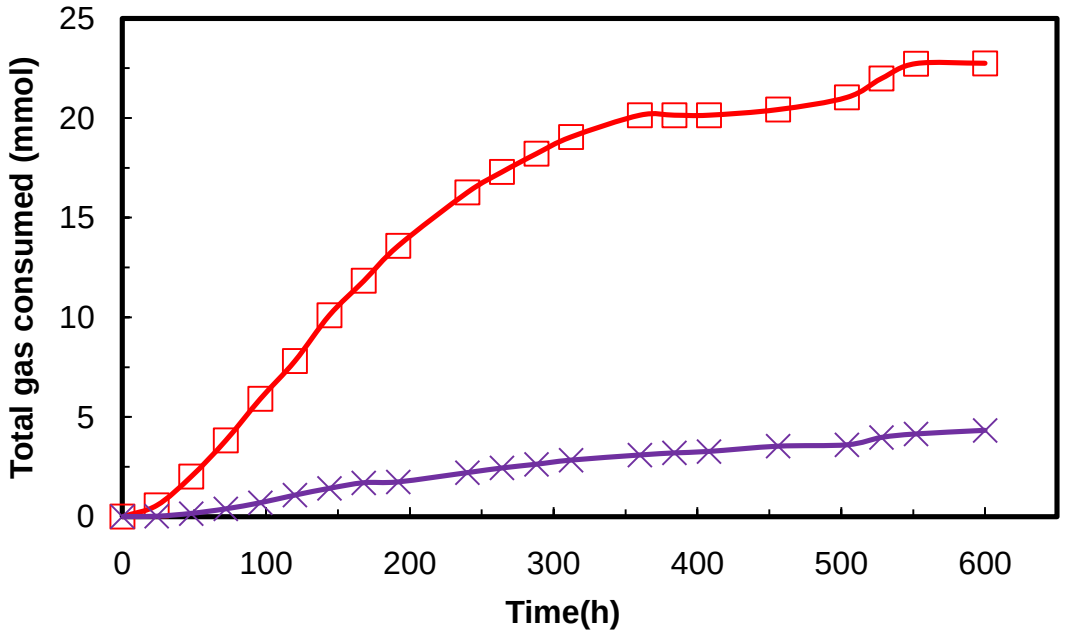

Figure 3.9 Total moles of CO and H_2 consumed by *Clostridium* strain P11 in 10 g/L corn steep liquor medium: (□) CO; (×) H_2.

In 10 g/L CSL medium, about 26% (5.9 mmol) of the total CO consumed by strain P11 was utilized for cell growth and acetic acid production in the first 96 h (Figure 3.2 and 3.9). It is clear that almost 62% of the total CO consumed was utilized between 96 and 380 h for ethanol and butanol production. During this period cells are in the stationary phase and need less carbon for energy and cell metabolism. The remaining 12% of the total CO consumed was utilized by the cells after 400 h (Figure 3.11) and also a small increase in acetic acid was measured. Nearly 16% of the total H_2 consumed was utilized for cell growth and acetic acid production in the first 96 h. The percentage of H_2

consumed after 168 h was about 61%, which may have been used by the cells for generating reducing equivalents necessary for ethanol and butanol production. Utilization of H_2 for generating reducing equivalents increased the efficiency of carbon conversion to alcohol (Ahmed and Lewis 2007).

In 20 g/L CSL medium, about 54% of the total CO consumed was utilized within the first 72 h for cell growth and acetic acid production (Figure 3.10). The remaining 46% were used for ethanol and butanol production. A small amount of H_2 was consumed during syngas fermentation in the 20 g/L CSL medium.

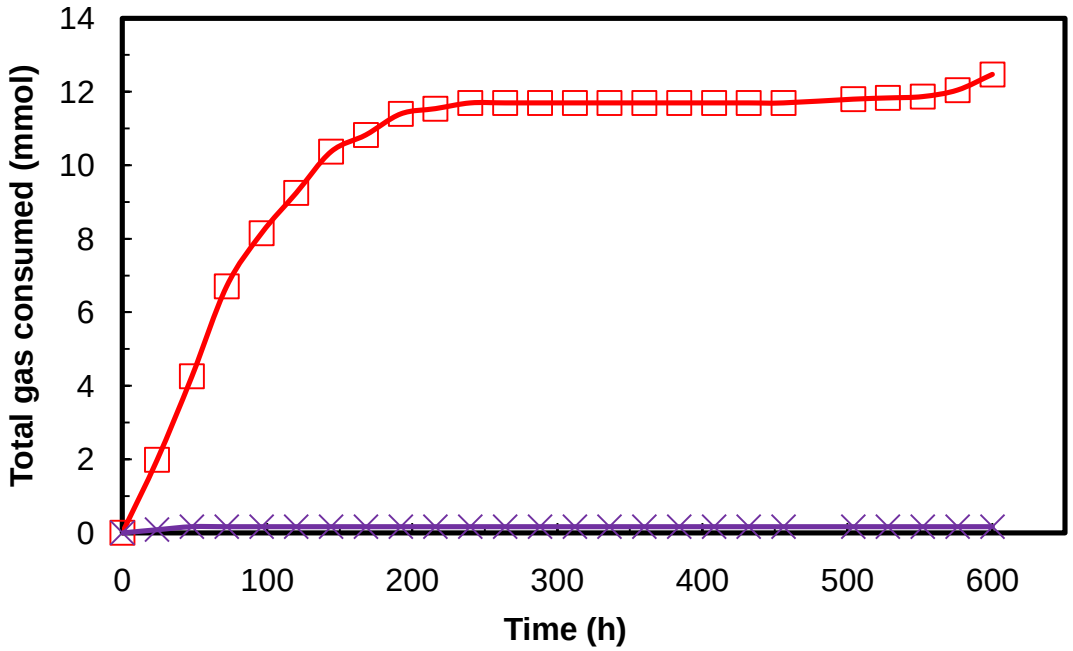

Figure 3.10 Total moles of CO and H_2 consumed by *Clostridium* strain P11 in 20 g/L corn steep liquor medium: (□) CO; (×) H_2.

The specific uptake rates (q_{CO} and q_{H2} in mmoles of substrate/min. g cells) of CO and H_2 in 10 g/L and 20 g/L CSL media are shown in Figures 3.11 and 3.12, respectively. More CO was consumed than H_2 in both media. In addition, the H_2 uptake was not

detected after 48 h in the 20 g/L CSL medium (Figure 3.12). This is different from what was observed in the 1 g/L YE (Figure 3.6) and 10 g/L CSL (Figure 3.11) media.

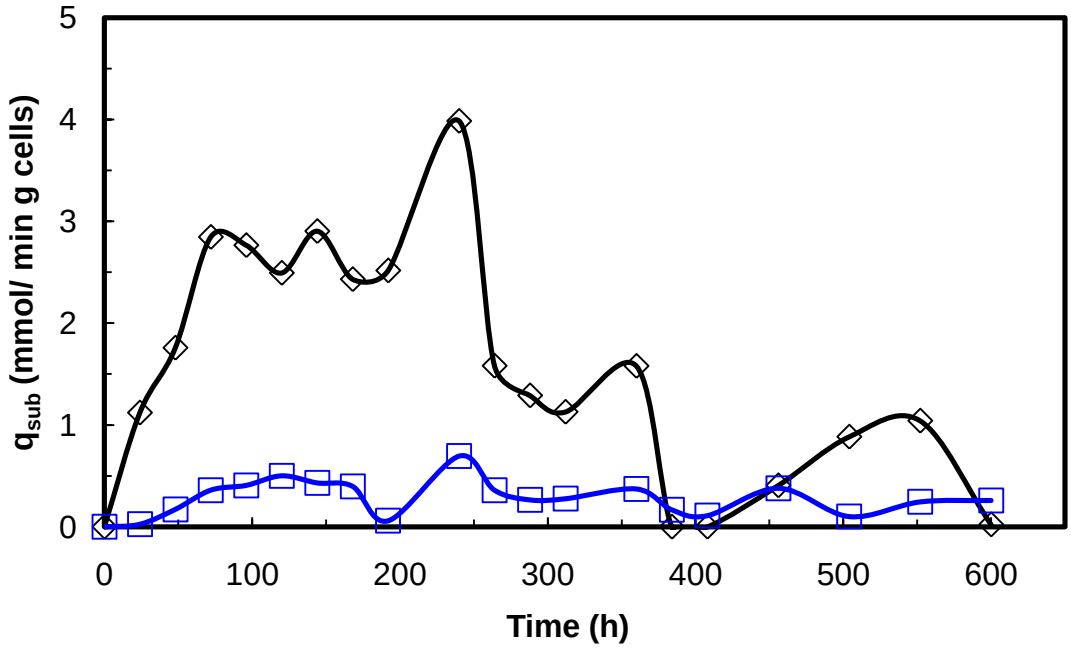

Figure 3.11 Specific uptake rates (q_{sub}) of CO and H_2 by *Clostridium* strain P11 in 10 g/L corn steep liquor medium: (◇) CO; (□) H_2.

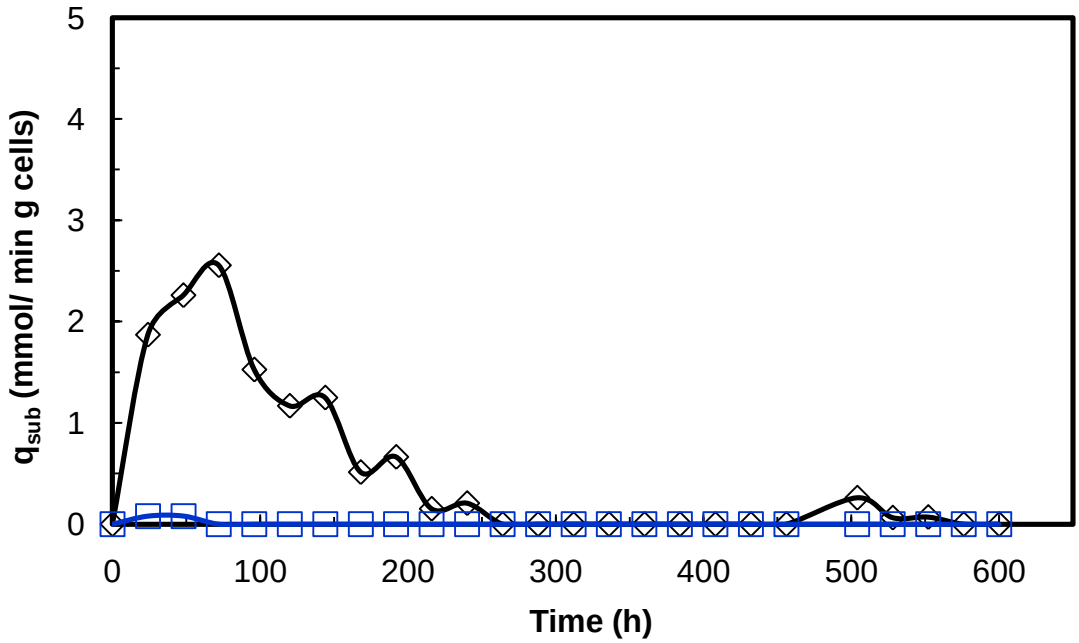

Figure 3.12 Specific uptake rates (q_{sub}) of CO and H_2 by *Clostridium* strain P11 in 20 g/L corn steep liquor medium: (◇) CO; (□) H_2.

In the 10 g/L CSL medium, both q_{CO} and q_{H2} increased during growth and acetic acid production. The CO and H_2 specific uptake rates in the 10 g/L CSL medium remained nearly constant between 72 and 192 h during which some ethanol was produced (Figure 3.2). After 240 h, the specific uptake rates of both CO and H_2 decreased and only small amounts of gases were consumed for cell maintenance and ethanol formation. The conversion efficiencies of CO and H_2 during the course of fermentation (600 h) in the 10 g/L CSL medium were 29 and 22%, respectively (Table 3.3). For the same fermentation time, ethanol and acetic acid yields from CO were 60% and 45% of the theoretical values, respectively.

The percentages of CO and H_2 conversion in the 20 g/L CSL medium during 600 h of syngas fermentation were 14% and 1%, respectively (Table 3.3). The ethanol and acetic acid yields from CO in the 20 g/L CSL medium at 600 h were 228% and 35%, respectively. Yields above 100% were likely due to the presence of some sugars in the CSL contributing to ethanol and acetic acid production. Also, it can be hypothesized that acetic acid could have been reduced to ethanol according to Equation 3.9. However, studies must be conducted to confirm the reaction.

$$CH_3COOH + 2H_2 \rightarrow C_2H_5OH + H_2O \quad (3.9)$$

According to the above equation, one mole of acetic acid is reduced to one mole of ethanol. It can be clearly shown that the yields of ethanol can be above 100% because of CO consumption. As hypothesized, subtracting the number of moles of ethanol produced due to possible conversion of acetic acid to ethanol between 24 h and 336 h, and between 24 h and 600 h, would give the moles of ethanol produced from CO. Final ethanol yields were recalculated using the obtained value after subtracting ethanol

produced from acetic acid. Based on modified calculation method, ethanol yields after 336 and 600 h were 46% and 129%, respectively (Table3.3).

The results demonstrate that strain P11 can grow autotrophically on syngas and produce acetic acid and ethanol. The specific growth rates of strain P11 in 1 g/L YE, 10 g/L CSL and 20 g/L CSL media were 0.059, 0.049 and 0.076 h^{-1}, respectively (Table 3.3). Though the specific growth rate of cells in the 1.0 g/L YE medium was 20% higher than in the 10 g/L CSL medium, 60% higher cell concentrations were obtained in media containing 10 g/L CSL and 20 g/L CSL (Figures 3.2 and 3.3). However, the higher final cell concentration and specific growth rate in the 20 g/L CSL medium compared to the 1 g/L YE medium could be due to the presence of additional nutrients such as sugars, water soluble vitamins, amino acids, minerals and trace metals in CSL (Liggett and Koffler 1948). The sugar content of the liquid portion of the crude CSL used in the present study is shown in Table 3.4. In a syngas fermentation study by Paneerselvam (2009) using strain P11 found that the specific cell growth rate of strain P11 grown on syngas (0.0327 h^{-1}) under similar conditions was lower compared to P11 grown on glucose concentrations at 6 g/L (0.0466 h^{-1}) and 12 g/L (0.0485 h^{-1}). However, in the current experiments the specific growth rates of strain P11 with syngas as the carbon source were higher than those obtained with glucose (about 0.0485 h^{-1}). This could be due to the change in metabolic characteristics of strain P11 over time.

Also, Amartey and Jeffries (1994) found that CSL is composed of 1.2% dextrose, 0.64% maltobiose, 4.0% maltotriose, 4.8% lactic acid, 0.06% acetic acid, and 0.35% glycerol. However, the composition of CSL changes from batch to batch and is confirmed by analyzing for sugars in CSL samples obtained from two different lots using

HPLC **(**Agilent 1100 series, Wilmington, DE**)** (Table 3.4). CSL from lot 117K0018 was used for the current research.

Table 3.4 Sugar composition of crude corn steep liquor - liquid portion.

Sugar	Concentration (g/L)	
	Lot #117 K0018	Lot # 018K0026
Cellobiose	33	33
Glucose	11	7
Xylose	5	5
Galactose	6	6
Arabinose	7	2
Mannose	3	3
Total sugars	65	56

The product formation by acetogens occurs in two different phases, namely the acidogenic phase and solventogenic phases (Diekert and Wohlfarth 1994). Similar to other acetogens, *Clostridium* strain P11 utilizes the reductive acetyl-CoA pathway for cell growth and product formation. Energy is generated in the form of ATP by the cells during the reduction of acetyl-CoA to acetate (acidogenesis). This suggests that acetic acid is growth related as was observed in all media used (Figures 3.1 to 3.3). Acetic acid consumption was also observed in both YE and CSL media. However, more acetic acid was consumed in 20 g/L CSL medium compared to 1 g/L YE medium.

The fermentation switched to solventogenesis in the stationary growth phase (Figures 3.1 to 3.3). However, negligible or no ethanol was produced during the growth

phase. The rate of ethanol production increased in the early stationary phase and decreased with the decline in cell concentration (Figures 3.1 to 3.3). Cells remained in stationary phase for about 400 h. However, ethanol production rates decreased as cells entered the death phase. After 600 h of fermentation, the ethanol and acetic acid concentrations in 1 g/L YE, 10 g/L CSL and 20 g/L CSL media were 1.3, 1.5, 2.7 g/L and 2.5, 2.3 and 0.81 g/L, respectively (Table 3.3). An approximate twofold increase in ethanol production was observed in 20 g/L CSL medium compared to YE and 10 g/L CSL media with the syngas (20% CO, 15% CO_2, 5% H_2 and 60% N_2). These results were different from those observed by Saxena (2008) with the same microorganism grown on a different gas mixture (70% CO, 24% N_2 and 6% CO_2). Strain P11 produced about 5.6 g/L of ethanol in both 1 g/L YE and 20 g/L CSL media (Saxena 2008). However, growth and product distribution varies with the composition of syngas used. Higher ethanol production in 20 g/L CSL can also be due to the presence of more nutrients. Significantly more butanol formation by strain P11 was observed in 10 g/L and 20 g/L CSL media compared to 1 g/L YE medium (Table 3.3.).

CO was preferably used by strain P11 compared to H_2 as seen from specific uptake rates (Figures 3.6, 3.11 and 3.12). Moreover, net consumption of CO_2 was not observed in any of the media used. Instead, CO_2 was produced during the fermentation (Figures 3.4, 3.7 and 3.8). This clearly indicates that strain P11 prefers CO as a primary carbon source compared to CO_2. The rate of CO consumption and CO_2 production were higher in the exponential and early stationary growth phases; whereas, consumption of H_2 was higher only during the early exponential phase and mainly during acetic acid production. This indicates that cells consumed CO to produce ethanol, acetic acid and

CO_2 according to Equations 3.1 and 3.3. A maximum CO consumption of about 60% was observed in 1 g/L YE and 10 g/L CSL media between 24 and 168 h. The same amount of CO was consumed during the period 24 and 96 h in 20 g/L CSL medium. Ethanol and acetic acid could have been produced from CO_2 and H_2 through Equations 3.2 and 3.4. However, our experimental results never showed consumption of CO_2, so it was difficult to determine yields of ethanol and acetic acid from CO_2. Cotter et al (2009) observed similar growth kinetics during syngas fermentation by *C. ljungdahlii*. The concentration of CO and H_2 in the head space decreased over time during growth phase with an increase in CO_2 concentration.

The maximum H_2 consumption of 75% was measured between 24 and 144 h in 1 g/L YE medium (Figure 3.4), while it was about 40% in 10 g/L CSL medium (Figure 3.7). However, H_2 consumption of about 1% was measured in the 20 g/L CSL medium (Figure 3.8). H_2 consumption substantially decreased after about 192 h in the 1 g/L YE medium. The decrease in H_2 consumption by the cells in all the media might be due to the inhibition of hydrogenase enzyme by high levels of CO. As the CO uptake rate decreased over time, the partial pressure of CO in the head space increased. CO at higher partial pressure was found to be a potential inhibitor of hydrogenase enzyme in *Clostridium acetobutylicum* (Kim et al. 1984). However, it is not known if this type of inhibition occurs in strain P11, which warrants further investigation. In the syngas mixture, both CO and H_2 serve as energy and electron sources. If H_2 is used as an electron source, then CO can be utilized for cell growth and production of reduced products. Cells would start utilizing CO as an electron source if there is not enough H_2 in the gas phase. This mechanism reduces the amount of carbon needed for ethanol production. Therefore,

increasing the concentration of H_2 in the gas phase would increase the amount CO routed towards ethanol production. Moreover, increased H_2 partial pressure improved alcohol yields from glucose by *C. acetobutylicum* (Yerushalmi et al. 1985).

The results in the present study demonstrate that CO conversion efficiencies were similar in both 1 g/L YE and 10 g/L CSL media, but was lower in 20 g/L CSL medium (Table 3.3). The reason for lower CO consumption in 20 g/L CSL medium could be due to the consumption of more acetic acid (Figure 3.3) by strain P11 compared to 1 g/L YE (Figure 3.1) and 10 g/L CSL (Figure 3.2) media.

To make syngas fermentation more economical, the fermentation medium used should be inexpensive and the process should be optimized in order to obtain high ethanol titers with high productivity. The feeding rate of syngas to P11 should also be controlled to increase syngas conversion efficiency while fermentation should be stopped when gas consumption and product formation rates decline. The cost of the various fermentation media used for syngas fermentation by strain P11 is summarized in Table 3.5. The total cost of the media is calculated based on Sigma Aldrich listed price as of November 2009. The cost of these media is high because of the cost of the MES buffer used, which accounts for 70% of the total media cost reported in Table 3.5. It can be seen that the 20 g/L CSL medium is about 26% less expensive than 1 g/L YE and 10 g/L CSL media. However, the cost of CSL when purchased in large quantities at $50 per ton (Lawford and Rousseau 1997) is over 500 times lower than its cost from Sigma Aldrich.

The amount of ethanol produced in 20 g/L CSL medium is 108% and 80% higher than in the 1 g/L yeast extract and 10 g/L CSL media, respectively (Table 3.3.). This demonstrates that 20 g/L CSL medium could be used as a lower cost alternative to yeast

extract medium in syngas fermentation. Moreover, more production butanol was noticed in CSL media. So using CSL at a concentration of 20 g/L has the potential to reduce the cost of ethanol production because it increases ethanol titer and productivity.

Table 3.5 Cost of various media used for syngas fermentation.

Media	Cost[a] ($/L)
Yeast extract[b] 1 g/L	$8.40
CSL[b] 10 g/L	$8.57
CSL[b] 20 g/L	$6.25
CSL[c] 10 g/L	$8.21
CSL[c] 20 g/L	$5.53

[a] MES buffer accounts for 70% of the total media cost
[b] Values based on Sigma-Aldrich on November 2009
[c] Values based on cost of CSL from Lawford and Rousseau (1997)

3.4 Conclusions

The effect of corn steep liquor (CSL) at two different concentrations (10 g/L and 20 g/L) on syngas fermentation by strain P11 in 250 mL serum bottles was compared with the 1 g/L yeast extract (YE) medium. The results clearly indicate that CSL can be used as a lower cost and alternate complex nutrient source in syngas fermentation compared to YE. The specific growth rate of strain P11 in 20 g/L CSL medium was 29% and 55% higher compared to 1 g/L YE and 10 g/L CSL media, respectively. Also, the amount of ethanol produced in 20 g/L CSL (2.7 g/L) was 108% and 80% higher than the 1 g/L yeast extract medium (1.3 g/L) and 10 g/L CSL medium (1.5 g/L), respectively.

Moreover, production of butanol (less than 0.6 g/L) by strain P11 was only measured in CSL media. Further optimization of media composition using CSL as the primary nutrient would significantly reduce the fermentation costs on an industrial scale.

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CHAPTER IV

EVALUATION OF CORN STEEP LIQUOR AS A SUPPLEMENT TO THE FERMENTATION MEDIUM IN ETHANOL PRODUCTION FROM SYNGAS USING *CLOSTRIDIUM* STRAIN P11 IN 7.5-L CSTR

4.1 Introduction

Global industrialization has led to increase in consumption of fossil fuels and natural resources. Over half of the gasoline required for the U.S. transportation and industrial sectors is imported from other countries (Demain et al. 2005). Fossil fuels are finite in resources; fluctuate in prices and are not environmentally friendly. Thus, there is a need to develop sustainable fuels such as bioethanol and biodiesel from renewable resources such as plant biomass (Huber et al. 2006). These biofuels are environmentally friendly but need some improvements in their production in order to make them economical and energy efficient. The use of biofuels could significantly reduce green house gas emissions from vehicles and also promote the economic status of the country (Balat 2008).

Bioethanol production from corn is a well established technology. However, the use of corn for bioethanol production would lead to competition for edible corn products, which would further lead to increase in food prices (Ahring et al. 1996). An

alternative for bioethanol production apart from corn milling is production of synthesis gas (syngas) from gasification of various non edible feedstocks such as biomass material, municipal solid wastes, bioenergy crops and crop and forest residues, and then fermentation of syngas to ethanol (Yang et al. 2009). Syngas is a mixture of CO, CO_2, H_2, CH_4 and N_2 with a few impurities such as tars and particulates. Syngas obtained from gasification of biomass can be fermented to ethanol using microbial catalysts at ambient temperature and pressure, thus making the process more advantageous over ethanol production using chemical catalysis (Najafpour and Younesi 2006; Vega et al. 1990b; Worden et al. 1991). Microorganisms containing biocatalysts can easily be reproduced by the addition of nutrients to the fermentation broth; whereas, regeneration of the poisoned and ash deposited chemical catalysts is a tedious process and requires replacement with a new batch of catalysts. Moreover, waste biocatalysts can be processed and used as a protein additive in cattle feed.

Several acetogens are found to be capable of metabolizing single carbon compounds to produce ethanol and other high molecular weight products via acetogenic fermentation (Worden et al. 1991; Zeikus et al. 1985). Syngas can be metabolized to ethanol and butanol by several microbial catalysts such as *Bacillus methylotrophicum* and *Clostridium ljungdahlii* (Vega et al. 1989; Worden et al. 1991).

Clostridium ljungdahlii (Phillips et al. 1994), *Clostridium autoethanogenum* (Abrini et al. 1994), *Clostridium carboxidivorans* (Rajagopalan et al. 2002), and *Clostridium* strain P11 (Frankman 2009; Panneerselvam 2009; Saxena 2008) can grow on syngas components to produce ethanol and acetic acid. These microorganisms utilize the reductive acetyl-CoA pathway, also known as the “Wood - Ljungdahl” pathway, for the

synthesis of acetyl-CoA, conservation of energy and growth. The acetyl-CoA formed during fermentation can be further reduced to ethanol and other products (Drake 1992). *Clostridium* strain P11 utilizes CO, H_2 and CO_2 for growth and production of acetic acid and ethanol.

There are major challenges in syngas fermentations, which include low cell yields and mass transfer limitation due to low syngas solubility in the fermentation medium (Vega et al. 1990a; Worden et al. 1997). In order to obtain high ethanol yield and productivity, it is necessary to achieve high cell concentration and lower the mass transfer limitations in the bioreactor.

Bioreactors that achieve high mass transfer rates and high cell densities are most suitable for syngas fermentation. Gas-liquid mass transfer rates can be increased using high gas and liquid flow rates, high agitation rates, high specific gas-liquid interfacial areas and by increasing gas solubility. Solubility of gases can be enhanced by increasing the pressure inside the reactor or by addition of solvents such as 1-octene in water, acetonitrile, and phenol (Henstra et al. 2007; Purwanto et al. 1996; Xuan et al. 2008). However, addition of organic solvents to syngas fermentation media could be inhibitory to the microorganisms used.

Continuously stirred tank reactors (CSTR) are most commonly used in syngas fermentations because of their high gas-to-liquid mass transfer rates. CSTR operated at high impeller speeds enhance the overall mass transfer coefficient (K_La). High impeller speeds break up large bubbles into small bubbles, increasing the specific gas-liquid interfacial area (Bredwell et al. 1999). However, this requires high power-to-volume ratios, which is not economical on an industrial scale. In addition, low rise velocities of

small bubbles increase the contact time between the gas bubble and the liquid phase. Moreover, increasing gas flow rates would increase superficial gas velocity and hence K_La, but for reactions involving sparingly soluble gases, high gas flow rates could result in low gas conversion efficiencies. Gas recycle can also improve conversion efficiencies (Bredwell et al. 1999).

The use of microspargers was found to provide better mixing and improve mass transfer compared to conventional spargers (Varma and Al-Dahhan 2007). Moreover the use of a multi-orifice ring sparger increased gas holdup distribution as well as reduced poor mixing zones in the a draft tube anaerobic bioreactor with a conical bottom surface (Varma and Al-Dahhan 2007). Also, pneumatically agitated bioreactors provided good mass transfer with less power-to-volume ratio compared to CSTR (Bredwell et al. 1999). A microsparger was used in the current study to improve the mass transfer between the syngas and the liquid medium.

Apart from reactor configurations, the fermentation medium also plays an important role in cell and product yields. Ethanol production can be made cost effective by optimizing various process steps during fermentation. One important factor is the fermentation medium. The development of an optimized, low cost fermentation medium containing all essential nutrients required for cell growth and product formation would reduce the overall cost of the fermentation process. Standard strain P11 medium is composed of yeast extract, vitamins, minerals, trace metals and reducing agent (Saxena 2008). Apart from the reducing agent, yeast extract is the most expensive component that substantially increases the cost of the fermentation medium on an industrial scale. Some inexpensive nutrients that could replace yeast extract are corn steep liquor (CSL),

hydrolyzed cotton seed flour, hydrolyzed soy flour and ethanol stillage (Witjitra et al. 1996). CSL is a complex nutrient rich in reducing sugars, vitamins, amino acids, proteins, minerals and metals such as copper, zinc, and iron (Witjitra et al. 1996). CSL is used as an essential nutrient for growing microorganisms and is also used for the production of ethanol by *Saccharomyces cerevisiae* (Kadam and Newman 1997) and *Zymomonas mobilis* (Lawford and Rousseau 1997) and butanol by *C. beijerinckii* (Parekh et al. 1999). CSL is lower cost nutrient compared to yeast extract. The incorporation of CSL at 20 g/L in the fermentation medium resulted in elimination of few growth factors from standard P11 medium (Saxena 2008). However, previous studies did not investigate growth and product kinetics of strain P11 in CSL media. In addition tests were done in 160 mL serum bottles with 10 mL of medium and only CO was fed to strain P11. The composition of syngas used in the above study is different from the syngas composition that potentially will be used for ethanol production on a large scale.

The main objective of this research was to explore growth and product kinetics during syngas fermentation by *Clostridium* strain P11 with three different media formulations in a 7.5-L CSTR pressurized to 143 kPa (absolute). Standard yeast extract and CSL media was used. Cell mass, ethanol, acetic acid and butanol concentrations, CO and H_2 utilization, and redox potential were measured.

4.2 Materials and methods

4.2.1 Experimental setup

All fermentations were carried out in a 7.5 L Bioflo 110 glass fermentor (New Brunswick Scientific Co,.Edison, NJ) with a 3 L working volume. The fermentor is equipped with an agitator, microsparger, baffles, pH probe, dissolved oxygen probe,

redox probe, external electrical heating jacket for maintaining temperature inside the reactor, peristaltic pumps for the addition of nutrients and solutions, including acid, base, and antifoam, ports for feeding nutrients and product removal, septum ports for addition of inoculum, sample port, and condenser connected to a chilled water line. Agitation was provided by three six-blade Rushton turbine impellers of 59 mm diameter located at an equal distance on a top-driven impeller shaft. Four baffles were symmetrically arranged to avoid vortex formation of liquid media and to improve mixing. A microsparger (New Brunswick Scientific Co,. Edison, NJ) with a pore size of 10-15 microns made of 316 SS material was used for gas sparging. The BioFlo 110 controller was connected to a computer installed with BioCommand® software to log real time data and monitor and control the fermentation process.

A bench top panel (Figure 4.1) was designed and instrumented with mass flow controllers (Model 100, Porter instrument Inc, Hatfield, PA), an oxygen scrubbing system connected to a temperature controller to heat the system for regeneration of copper catalyst with H_2, multi-parameter M400 transmitter (Mettler Toledo, Columbus, OH), back pressure regulators, thermocouples connected to a temperature indicator for measuring temperature of the gas entering and leaving the oxygen scrubber and reactor, and pDAQ (Iotech, Cleveland, Ohio) to log in gas inlet and outlet temperatures and oxidation-reduction data. The process lines from the panel are connected to the reactor ports using silicone tubing connected with 0.2 µm PTFE membrane filters (VWR International, West Chester, PA) to filter sterilize the syngas entering and leaving the reactor.

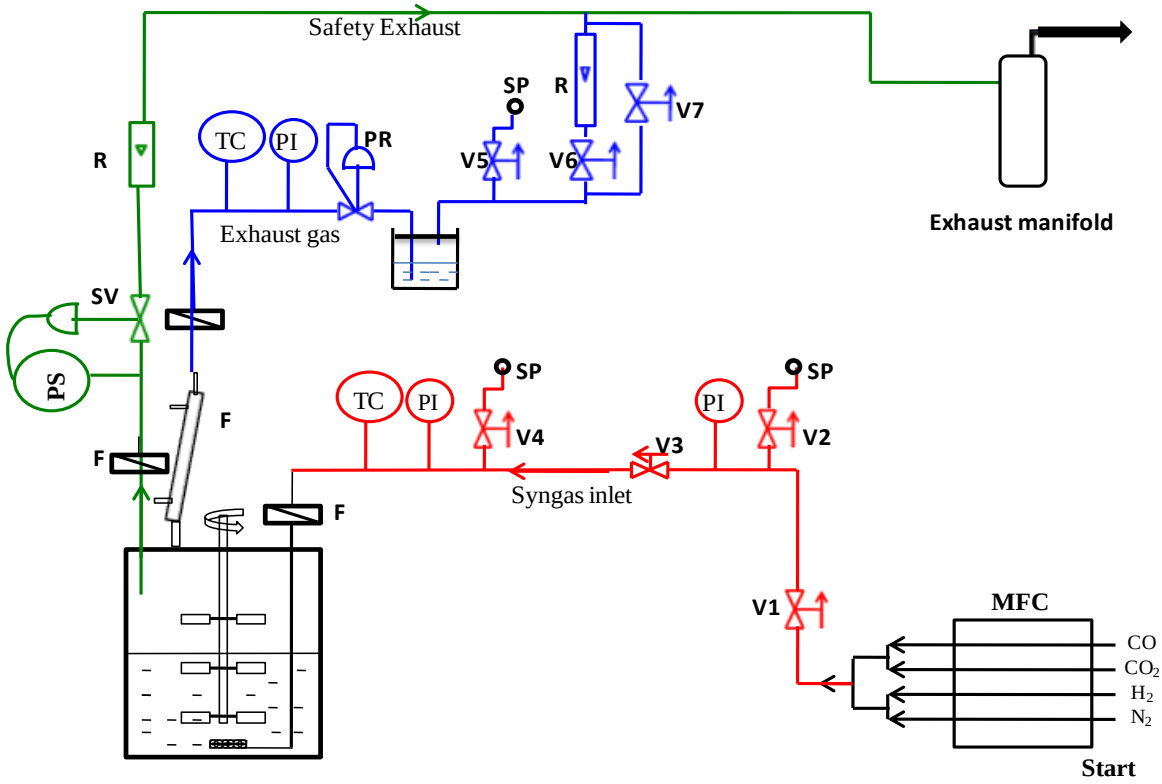

Figure 4.1 Schematic of 7.5-L fermentor instrumented with mass flow controller (MFC), pressure gauge (PI), pressure switch (PS), thermocouple (TC), gas sample port (SP), back pressure regulator (PR), two-way valve (V1 to V7), 0.2 micron PTFE membrane filter (F), solenoid valve (SV), rotameter (R).

Gases leaving the fermentor pass through a condenser cooled by circulating 10% ethylene glycol-water solution chilled to 5°C using a refrigerated circulator (1156 D, VWR International, West Chester, PA). Condensed vapors return to the fermentor. Uncondensed vapor from the condenser flows with the syngas through a bubble trap filled with 150 mL deionized water maintained at 10°C using chilled water. The bubble trap captured ethanol that was not condensed in the condenser.

The design (Figure 4.1) allows operating the fermentor at various pressures and also recycling exhaust syngas back into the fermentor at various ratios. However, for the current experiments, gas recycling was not used. A safety exhaust line with a solenoid valve connected to a pressure switch was connected to the fermentor. The pressure switch was set at 153 kPa absolute.

The oxidation-reduction potential (ORP) was measured using an autoclavable redox probe with an Ag/AgCl reference electrode (InPro3250/325/PT100, Mettler Toledo, Columbus, OH) connected to a multi-parameter M400 transmitter (Mettler Toledo, Columbus, OH) with a VP6 cable. The ORP values were displayed on the transmitter and an analog output (range 4-20 mA) from the transmitter was connected to a USB port data acquisition system, pDAQ 56 (Iotech, Cleveland, Ohio). ORP was measured in terms of milli volts (mV), so a 250 ohms resistor was connected on the input pins of the DAQ system to convert the transmitter analog output from 4-20 mA to 1-5 V. The redox value from Ag/AgCl reference electrode is converted to standard hydrogen electrode (SHE) by the addition of +222.4 mV to the value from the Ag/AgCl electrode (Kakiuchi et al. 2007). For example, if the Ag/AgCl reference electrode reads a value of

-300 mV, then the value in SHE is -77.6 mV. The ORP probe was calibrated before each run using + 220 mV standard redox buffer from Mettler Toledo.

4.2.2 Microorganism

Clostridium strain P11 (ATCC PTA-7826) provided by Dr. Ralph Tanner, University of Oklahoma, was used. Strain P11 is a gram positive, rarely motile, rod shaped bacterium and occur singly or in chains (Saxena 2008). It can grow autotrophically on CO/CO_2 or H_2/CO_2 gas mixtures. The optimum temperature and pH for strain P11 were 37°C and 6.10, respectively.

In order to reduce the lag phase, viable cells are inoculated into the bioreactor. Cells from the stock inoculum were passaged twice prior to inoculation into the reactor. Cell passaging was performed in 250 mL serum bottles with 100 mL working volume. The first passage was inoculated with 10% (v/v) of inoculum and then purged with syngas (20% CO, 15% CO_2, 5% H_2 and 60% N_2) at 239 kPa (absolute). When the OD measured at 660 nm in the first passage reached 0.5 units, cells were transferred to a second passage. Finally, cells from the second passage were transferred to the fermentor at 10% (v/v) when the OD reached 0.5 units. For a 3-L working volume, 300 mL of inoculum were required. A total of five 250-mL serum bottles with 100 mL of inoculum were prepared. Inoculum was prepared in excess to ensure adequate supply of inoculum that reached OD of 0.5.

4.2.3 Fermentation media

Strain P11 is highly sensitive to oxygen and hence the fermentation was performed completely under strict anoxic conditions. Three different fermentation media composition optimized by Saxena (2008) were used in the current study (Table 4.1).

Table 4.1 Composition of various media used for syngas fermentation.

	Standard-Yeast extract 1 g/L	CSL 10 g/L	CSL 20 g/L
Yeast extract (g)	1	-	-
Corn steep liquor (g)	-	10	20
Minerals (mL)	30	30	-
Trace metals (mL)	10	10	10
Vitamins (mL)	10	10	-
MES (g)	10	10	10
0.1% Resazurin (mL)	1	1	1
Ammonium chloride (g)	-	-	1.25
4% Cysteine sulfide (mL)	10	10	2.5
Total volume, (L)	1	1	1

The standard fermentation medium contained yeast extract, minerals, trace metals, vitamins and reducing agents. The mineral stock solution contained (per liter) 100 g ammonium chloride, 4 g calcium chloride, 20 g magnesium sulfate, 10 g potassium chloride and 10 g potassium phosphate monobasic. The composition of the trace metal stock solution (per liter) was 0.2 g cobalt chloride, 0.8 g ferrous ammonium sulfate, 1 g manganese sulfate, 0.2 g nickel chloride, 2 g nitrilotriacetic acid, 0.02 g sodium molybdate, 0.1 g sodium selenate, 0.2 g sodium tungstate and 1 g zinc sulfate. The stock vitamin solution (per liter) contained 0.005 g of p-amino benzoic acid, 0.002 g d-biotin,

0.005 g pantothenic acid, 0.002 g folic acid, 0.01 g 2-MercaptoEthane Sulfonate Sodium (MESNA), 0.005 g nicotinic acid, 0.01 g pyridoxine, 0.005 g riboflavin, 0.005 g thiamine, 0.005 g thioctic acid and 0.005 g vitamin B-12. Unless mentioned all media components were purchased from Sigma Aldrich (Saint Louis, MO). Yeast extract (Difco laboratories, Detroit, MI) and corn steep liquor (Sigma Aldrich, Saint Louis, MO) were used as complex nitrogen and nutrient sources in the fermentation media (Table 4.1). Antifoam B Emulsion (Sigma Aldrich, Saint Louis, MO) at a concentration of 10% v/v was used to control foam formation inside the fermentor. About 0.2 mL of sterilized antifoam solution was added when the height of the foam in the fermentor was two inches above the surface of the medium.

In order to produce consistent results, CSL from the same batch was used throughout the studies. Crude CSL contains about 50% solids. Before the addition of CSL into the medium, solids from crude CSL were removed by centrifugation at 13000 rpm for 10 min. Resazurin solution (0.1%) was added as a redox indicator. Morpholinoethanesulfonic acid (MES) was added as a biological buffer to prevent excessive fluctuations in the pH during the course of the fermentation. The initial pH of the medium was adjusted to 6.05 before inoculation. The sugar composition of the liquid portion of crude CSL was analyzed using HPLC **(**Agilent 1100 series, Wilmington, DE**)** with refractive index detector. The column used was Aminex HPX 87P (Bio-Rad, Sunnyvale, CA, USA). The column was operated at 85°C with de-ionized water as the mobile phase pumped at 0.6 mL/min for 30 min per sample. The concentration of sugars analyzed is shown in Table 4.2.

Table 4.2 Sugar composition in liquid portion of crude corn steep liquor used in syngas fermentation.

Sugar	Concentration (g/L)
Cellobiose	33
Glucose	11
Xylose	5
Galactose	6
Arabinose	7
Mannose	3
Total sugars	65

Elemental composition of yeast extract and CSL media were determined using Inductively Coupled Plasma - Atomic Emission Spectroscopy (ICP-AES) (Spectro Analytical Instruments Inc. NJ, U.S.A). The elemental compositions of the solid and liquid portions of crude CSL are shown in Table 4.3. The elemental compositions of 1 g/L yeast extract, 10 g/L CSL and 20 g/L CSL media used in this study are shown in Table 4.4. The elemental composition analysis was done to have better understanding of the effect of the concentrations of trace elements and minerals on the characteristics of strain P11 during syngas fermentation.

Table 4.3 Elemental composition of solid and liquid portions of crude corn steep liquor.

Element	Concentration (ppm)	
	Solid	Liquid
B	ND	9
Al	ND	2
Mn	54	17
Co	0	0
Ni	4	4
Cu	58	1
As	1	1
Se	ND	ND
Sr	12	1
Mo	3	1
Ba	17	0
W	17	0
Zn	148	26
Fe	231	63
Ca	12392	15
Mg	10605	1530
Na	659	9650
K	1360	21320
P	2202	23250
S	8998	13040
Cr	4	3
Cl	1475	5897
Total N	76452	15760
Total C	492000	295000

ND: not detected (below detection limits)

Table 4.4 Initial concentrations of the elements present in yeast extract and corn steep liquor media used.

Element	Concentration (ppm)		
	YE (1 g/L)	CSL (10 g/L)	CSL (20 g/L)
Ba	0.009	0.009	0.006
As	0.021	0.052	0.016
Cu	0.038	0	0.002
Sr	0.043	0.053	0.024
Al	0.020	0.000	0.000
Cr	0.012	0.28	0.042
Mo	0.077	0.104	0.094
B	0.865	0.500	0.851
Ca	30.366	28.776	0.374
Se	0.401	0.422	0.398
Co	0.463	0.483	0.439
Ni	0.507	0.570	0.575
W	1.258	1.475	1.179
Fe	0.945	1.125	1.660
Zn	0.063	0.855	2.305
Mn	3.105	2.995	3.078
Mg	58.061	70.604	25.433
Na	91.5	203.1	185.0
S	5110.3	6139.2	11790.0
K	1369.3	2573.9	1738.2
P	75.0	202.5	234.4
Cl	2154.0	2157.0	916.6
Total N	500.0	53000.0	1397.5
Total C	12800.0	14900.0	6623.7

4.2.4 Syngas

Commercial syngas with gas composition similar to producer gas generated from our gasification facility was used in this study. Syngas was composed of 20% CO, 15% CO_2, 5% H_2 and 60% N_2 (by volume).

4.2.5 Fermentor operation

Before medium preparation, the pH and redox probes were calibrated. A two point calibration was done on pH probes using pH 7 and pH 4 buffer solutions. At first, the probe is inserted in pH 7 buffer and the set point on the control panel is set to 7.0 when the raw input stabilized. Similarly the set span value was set to 4.0 following the same procedure using pH 4 buffer. A one point calibration using 468 mV redox buffers was done on redox probe. All media components in (Table 4.1) except cysteine sulfide were added into the fermentor to a total volume of 3 L by adding appropriate amount of deionized water. The pH of the media was adjusted to 6.1 before sterilization using 5 N KOH. After adjusting the pH, all reactor components including head plate were assembled and all probes (pH, redox and DO) were inserted into the fermentor. It is very important to ensure that the o-rings and gaskets in the head plate ports are not damaged to avoid leaks during sterilization.

Before sterilization, the fermentor was checked for leaks by slightly pressurizing with compressed N_2, with exhaust lines closed. Snoop solution was used to check for leaks in the fermentor. Once no leaks were found in the fermentor, the fermentor containing the medium was autoclaved at 121°C and 205 kPa (absolute) for 20 min. The exhaust line from the condenser of the fermentor was kept partially open to prevent explosion of the glass vessel during sterilization.

The exhaust line was closed immediately after sterilization in order to prevent contamination of the media with oxygen. All ports, knurled adapters and nuts on the fermentor head plate were finger tightened again and probes were connected to the control unit. PTFE membrane filters, 0.2 µm, (VWR International, West Chester, PA) were installed on each gas inlet and outlet lines to prevent medium contamination and also to sterilize incoming syngas to the fermentor. The condenser was connected to a chiller with water circulation at 5°C to prevent the loss of medium and ethanol from the fermentor.

The maximum pressure rating of the glass fermentor is 170 kPa (absolute). However for safe operation, the pressure inside the fermentor was controlled at 143 kPa (absolute) using a back pressure regulator (Figure 4.1). The pressure switch on the safety exhaust line was set to 153 kPa (absolute), so that pressure switch triggers the solenoid valves open whenever the pressure increases above the set point.

The temperature of the medium was controlled at 37°C using an electrical heating jacket. The agitator speed in the fermentor was set at 150 rpm and the medium was purged with N_2 at 200 standard cubic centimeters per minute (sccm) for 24 h to remove any dissolved O_2 before the start of the syngas fermentation. Before switching from N_2 to syngas in the fermentor, the DO probe was calibrated by setting the "set zero" reading to zero. Then, syngas at 150 sccm was switched on. The volumetric syngas flow rate per unit liquid volume (vvm) was 0.05 min^{-1}.

The syngas was allowed to saturate the medium in the fermentor by purging for 2 h and then 4% cysteine sulfide solution was added as in Table 4.1. The medium was then inoculated with 10% (v/v) of cells in early or mid exponential phase from passage

two. A sample from the fermentor was collected before inoculation for metal and trace element analysis using ICP-AES. The Biocommand plus and pDAQ software were started immediately after inoculating the fermentor for data acquisition.

For the first 48 h, multiple gas and liquid samples were withdrawn every 4 h to 6 h interval from the fermentor to have more data to calculate specific growth rate for strain P11. Later, gas and liquid samples were collected every 24 h for a total of 360 h. Gas samples were withdrawn using 100-µL gas tight sample lock syringes (Hamilton Company, Reno, Nevada) from the sample port installed in the line after the bubble trap (Figure 4.1). Liquid samples of 25 mL were withdrawn from the fermentor under aseptic conditions. About 0.5 mL of the sample was used to measure cell mass concentration, while the remaining sample was filtered through a 0.2-micron nylon filter. From the filtrate, 2 mL of sample was collected for solvent analysis on GC and the remaining liquid sample was frozen for elemental analysis using ICP-AES.

4.2.6 Analytical procedures

4.2.6.1 Cell concentration

Cell concentrations were determined using a UV-Vis spectrophotometer (Varian Inc., Palo Alto, CA). Cell samples were collected in 2-mL cuvettes from the fermentor and the optical density (OD) was measured at 660 nm. Samples with OD above 0.4 were diluted so that the OD was within the linear range of the calibration curve, which is from 0 to 0.4. One OD unit corresponds to 0.34 g/L (Panneerselvam 2009).

4.2.6.2 Solvent analysis

Liquid samples were centrifuged at 13,000 rpm for 13 min. The supernatant was filtered through 0.45-µm nylon membrane filters (VWR International, West Chester, PA)

and frozen until further analysis. Ethanol, acetic acid and butanol were analyzed using a 6890 Gas Chromatography (GC) (Agilent Technologies, Wilmington, DE). A PoraPak QS 80/100 **(**Alltech, Deerfield, IL) packed column connected to a flame ionization detector (FID) was used for solvent analysis. The GC was operated under isothermal conditions with the oven temperature set constant at 210°C. Helium was used as a carrier gas with a flow rate set at 25 mL/min. Chromatograms were analyzed using CHEMSTATION® Data analysis package. The percentages of error in ethanol and acetic acid measurements were below 5%.

4.2.6.3 Gas analysis

GC, equipped with thermal conductivity detector (TCD), was used for gas analysis. A capillary column, Carboxen 1010 PLOT, (Supelco, Bellefonte, PA) was used to measure the composition of syngas components CO, CO_2, H_2 and N_2. Gas analysis was conducted in duplicate for each experiment. Argon was used as a carrier gas with an initial gas flow rate of 0.4 mL/min for the first 12 min, and then it was increased at a rate of 0.1 mL/min until it reached 0.8 mL/min. The oven temperature was set at 32°C for 12 min, after which the temperature was increased at a rate of 30°C per min until it reached 236°C. The temperatures of the column inlet and detector were set at 200°C and 230°C, respectively. Chromatograms were analyzed using CHEMSTATION® Data analysis package. The GC was calibrated by injecting different volumes of known concentrations of gas samples. Standards gas mix samples were frequently injected to check the accuracy of the calibration curves prepared. The percentages of error in CO, CO_2, H_2 and N_2 measurements were lower than 5%.

4.3 Results and discussion

4.3.1 Fermentation in 1.0 g/L yeast extract medium

The growth and product profiles of strain P11 in 1 g/L yeast extract medium are shown in Figure 4.2. Cells experienced a lag phase of 4 h, and then their concentration inside the fermentor increased exponentially from 0.02 g/L to 0.28 g/L in 44 h. The maximum specific growth rate was 0.055 h^{-1} during the first 44 h of fermentation. Then, cells entered deceleration phase during 44 to 72 h, followed by a stationary phase. The cell concentration was about 0.5 g/L during the stationary phase until 192 h. Cell concentration then decreased to 0.3 g/L and remained at this level from 216 h to 360 h of fermentation. The maximum cell concentration in the 1.0 g/L YE medium was 0.53 g/L, which was obtained after 168 h of fermentation. This is double the cell concentration noticed in the same medium during syngas fermentation in 250-mL serum bottles (Figure 3.1). This is due to the fact that 12 times more syngas was fed to the 7.5 L fermentor compared to the serum bottles.

The pH decreased from 6.10 to 4.59 in the first 72 h of incubation due to acetic acid production (Figure 4.2) because cells produce acetic acid during their growth. In addition, a small amount of ethanol production was also observed during the late stage of cell growth after 48 h of fermentation. A complete shift from acidogenesis to solventogenesis was observed after about 72 h. Ethanol production rate was highest between 72 to 168 h. During this period, the pH of the medium increased from 4.59 to 5.0 due to consumption of acetic acid by strain P11. During the solventogenic phase pH continued to increase and reached 5.21 by the end of the fermentation. The disappearance

of acetic acid from the fermentation medium is probably due to conversion to ethanol by strain P11 according to Equation 3.9.

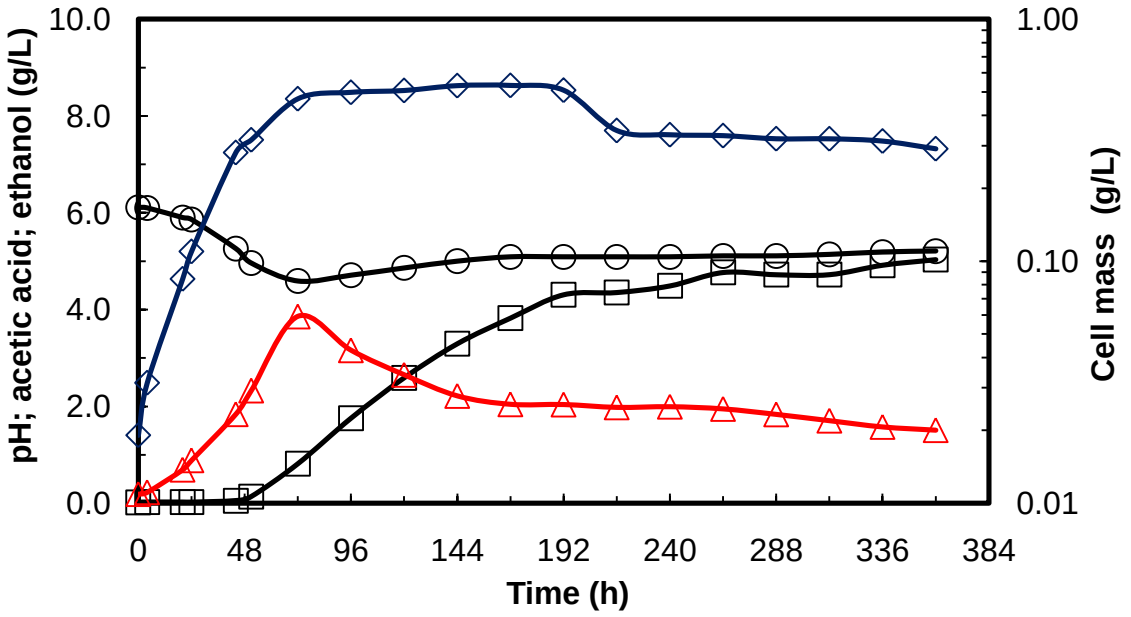

Figure 4.2 Growth, pH and product profiles using *Clostridium* strain P11 in 1 g/L yeast extract medium: (◇) cell mass; (□) ethanol; (Δ) acetic acid; (O) pH.

Ethanol and acetic acid were the major products during syngas fermentation. Acetic acid production is associated with a release of ATP by substrate level phosphorylation and hence it is a growth related product (Henstra et al. 2007). Acetic acid was predominantly produced during the growth phase with a maximum concentration of 3.86 g/L that corresponds to a productivity of 4.2 mg/Lh. However, acetic acid concentration was 1.81 g/L at the end of fermentation. Ethanol concentration increased from 0.05 to 3.83 g/L between 44 and 192 h. After 192 h, ethanol production rate decreased. The maximum ethanol concentration in the fermentor was 5.03 g/L after 360 h with a corresponding productivity of 16.9 mg/Lh. Moreover, some ethanol was not condensed and exited the condenser with the syngas. The uncondensed vapors of ethanol

in the exhaust gas stream were condensed in a water trap that was maintained at 10°C (Figure 4.1). At the end of the fermentation, the concentration of ethanol in the water trap reached 13 g/L. The initial and final volumes of water in trap were 150 mL and 135 mL, respectively. Taking into consideration the amount of ethanol in the fermentor, water trap and collected samples, the final ethanol concentration in the fermentor was 6.1 g/L, which corresponds to ethanol yield of 53% of the theoretical value based on CO consumption (Table 4.5). Ethanol yield was calculated based on CO consumption because insignificant amounts of H_2 were consumed during ethanol production. Ethanol concentration observed in the 7.5-L fermentor was over four times higher than in 250-mL serum bottles as shown in Figure 3.1.

During the first 72 h of fermentation in 1 g/L YE medium, CO, CO_2 and H_2 were utilized for cell growth and production of acetic acid (Figure 4.3). However, only CO and H_2 were consumed during fermentation in 250-mL serum bottles (Figure 3.4).

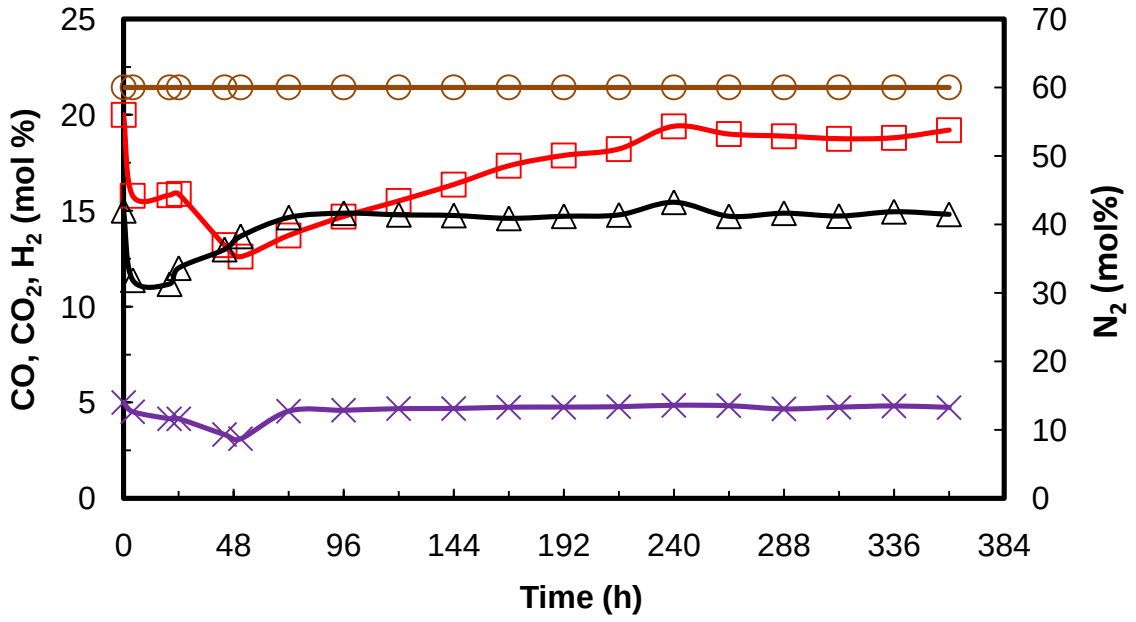

Figure 4.3 CO, CO_2, H_2 and N_2 profiles using *Clostridium* strain P11 in 1 g/L yeast extract medium: (□) CO; (Δ) CO_2; (×) H_2; (O) N_2.

In both of the 250-mL serum bottles and 7.5-L fermentor, growth and ethanol formation was more favored from CO than H_2. Similar conversion efficiencies of CO and H_2 (about 30%) were obtained in the fermentor. Around 18% of the CO_2 supplied to strain P11 was utilized during the first 44 h of fermentation. CO_2 could have been used in building cellular material and for acetic acid production according to Equation 3.4. When cells entered stationary phase, the rate of CO consumption slightly decreased, whereas CO_2 and H_2 consumption almost ceased. CO utilization by strain P11 continued until 360 h of fermentation i.e., before stopping the experiment. However the specific CO consumption rate was reduced along with the decrease in cells activity. This implies that cells in stationary phase utilized CO for cell maintenance and ethanol production (Figures 4.2).

The total moles of CO and H_2 consumed by strain P11 during the fermentation were 4.3 and 0.45 moles, respectively (Figure 4.4). The percentages of CO and H_2 consumed during the growth phase were about 39% and 55% of the total CO and H_2 consumed by strain P11, respectively. The conversion efficiencies of CO and H_2 after 360 h of fermentation were 11 and 5 %, respectively (Table 4.5). These conversion efficiencies were lower than in 250-mL serum bottles (Table 3.3) because of the high flow rate of syngas fed to the fermentor.

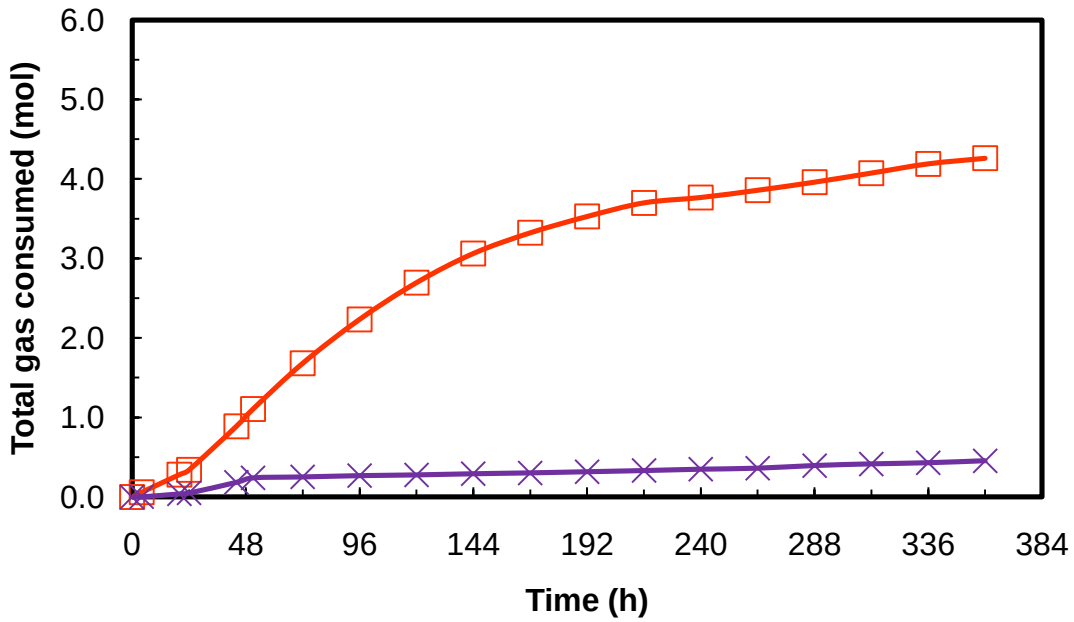

Figure 4.4 Total moles of CO and H_2 consumed by *Clostridium* strain P11 in 1 g/L yeast extract medium: (□) CO; (×) H_2.

The oxidation-reduction potential (ORP) or redox potential in standard hydrogen electrode (SHE) in the fermentation medium with strain P11 is shown in Figure 4.5. The redox potential profile is correlated to cell growth and product formation. The ORP decreased sharply from -96 to -206 mV (SHE) in the first 42 h after inoculation. Cell growth was observed when the ORP value was between -90 and -200 mV (SHE). The initial drop in redox potential was observed during lag phase of aerobic fermentations (Kwong et al. 1992; Tae Ho et al. 1998). During the stationary growth phase, the ORP values increased from -199 to -156 mV (SHE). About 85% of the total ethanol produced by strain P11 was observed when the ORP value was between -145 and -200 mV (SHE).

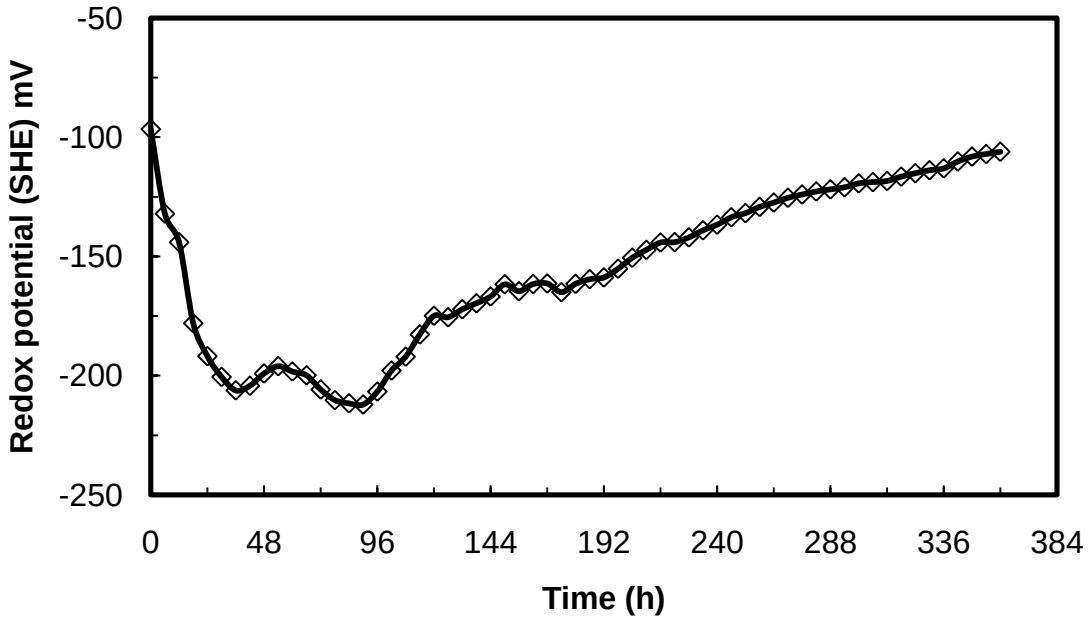

Figure 4.5 Redox potential profile of *Clostridium* strain P11 in 1 g/L yeast extract medium.

The key enzymes involved in the acetyl-CoA pathway are formate dehydrogenase (FDH), bifunctional carbon monoxide dehydrogenase/acetyl-CoA synthase (CODH/ACS) and hydrogenase are metalloenzymes (Drake 1994). These enzymes are metal dependant and consist of active sites with metals. In acetogens, FDH is a protein containing iron (Fe), selenium (Se), tungsten (W) and/or molybdenum (Mo) (Andreesen et al. 1974), where as CODH is nickel (Ni) and iron-sulfur (Fe-S) containing protein (Ragsdale et al. 1983). Hydrogenase contains Fe and/or Ni in their active site (Vignais et al. 2001). Based on the enzymes involved in the pathway, Mo, Ni, Se, W, Co, Zn and Fe were selected as important metals necessary the cellular activity.

The concentrations of trace metals and minerals in 1 g/L YE medium during syngas fermentation with strain P11 are shown in Figure 4.6. Around 61% of the Fe (Figure 4.6 c) supplied was consumed during growth phase (between 0 and 72 h), the

activity of hydrogenase is expected to be high during this phase because of the measured consumption of H_2, which was at its maximum rate during this phase (Figure 4.3). Also, 17% of Mo (Figure 4.6 b) was consumed during the growth phase. However, no significant change in the concentration of other metals (Ni, Se, W, Co, and Zn) was detected during the fermentation (Figure 4.6). It can be seen that very little amount of trace metals and minerals were consumed and there was no defined trend in elemental uptake by strain P11. This also shows that the trace metals and minerals were not depleted from the fermentation medium, which indicates that enough trace metals were supplied in the medium. The concentration profiles of other trace metals and elements as well as total nitrogen and carbon during the fermentation are available in Appendix A

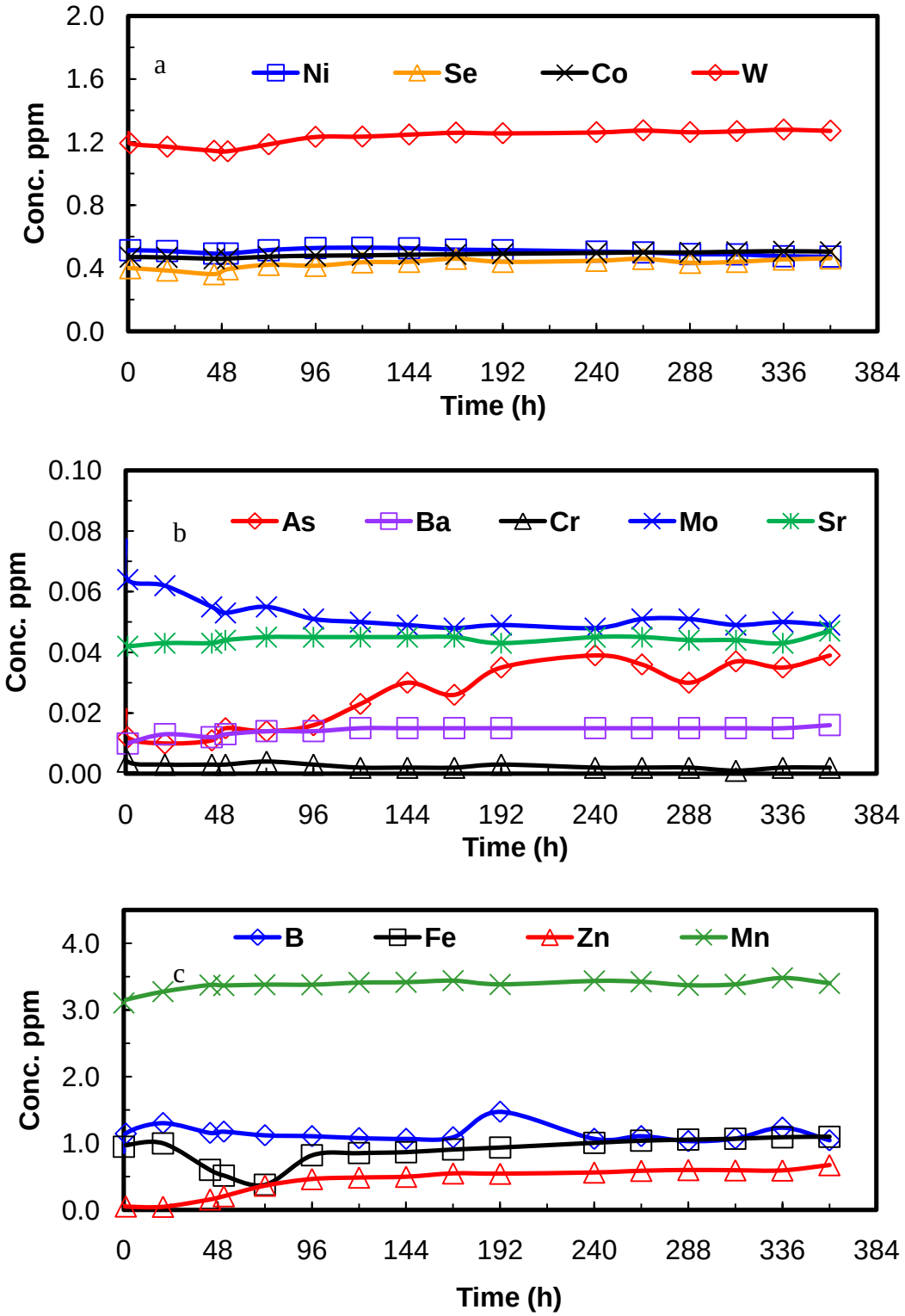

Figure 4.6 Trace metals and minerals profiles during syngas fermentation by strain P11 in 1 g/L yeast extract medium.

Table 4.5 Fermentation characteristics of *Clostridium* strain P11 in yeast extract and corn steep liquor media.

	Medium		
	YE 1 g/L	CSL 10 g/L	CSL 20 g/L
Fermentation time (h)	360	360	360
μ_{max} (h^{-1})	0.055	0.061	0.075
Final pH	5.21	5.11	4.85
Max. acetic acid (g/L)	3.9[a]	4.2[a]	4.0[b]
Acetic acid (g/L)	1.5	2.4	3.4
Amount of acetic acid consumed (g/L)	2.4	2.0	0.9
Ethanol (g/L)	6.1	8.6	9.6
Ethanol productivity[c] (mg/Lh)	17.0	24.0	27.0
Acetic acid productivity[c] (mg/Lh)	4.2	6.6	9.4
Ethanol yield from CO (% of theoretical value)	53	57	60
Acetic acid yield from CO (% of theoretical value)	8	9	12
Conversion efficiency CO, %	11	15	15
Conversion efficiency H_2, %	5	7	5

[a] Value at 72 h

[b] Value at 120 h

[c] Values at 360 h

4.3.2 Fermentation in 10 g/L corn steep liquor medium

The growth and product profiles of strain P11 in 10 g/L CSL medium are shown in Figure 4.7. After 4 h of lag phase, the cell concentration increased exponentially at a specific growth rate of 0.061 h^{-1} and reached a concentration of 0.29 g/L after 54 h of incubation. The exponential phase was followed by a deceleration phase for about 6 h. During the deceleration phase, the cell concentration increased to 0.45 g/L. Cells then reached stationary phase with an average concentration of 0.52 g/L. After 264 h, the cell concentration declined to 0.17 g/L by the end of fermentation. The drastic decrease in cell concentration was due to foam formation, which caused the cells to stick onto the wall of the fermentor. Foam formation in the medium was due to the proteins present in the medium and proteins released from dead cells (Doran 2006). The maximum cell concentration in the medium with 10 g/L CSL was 0.55 g/L after 192 h of cultivation. This is similar to the maximum cell concentration obtained in YE medium.

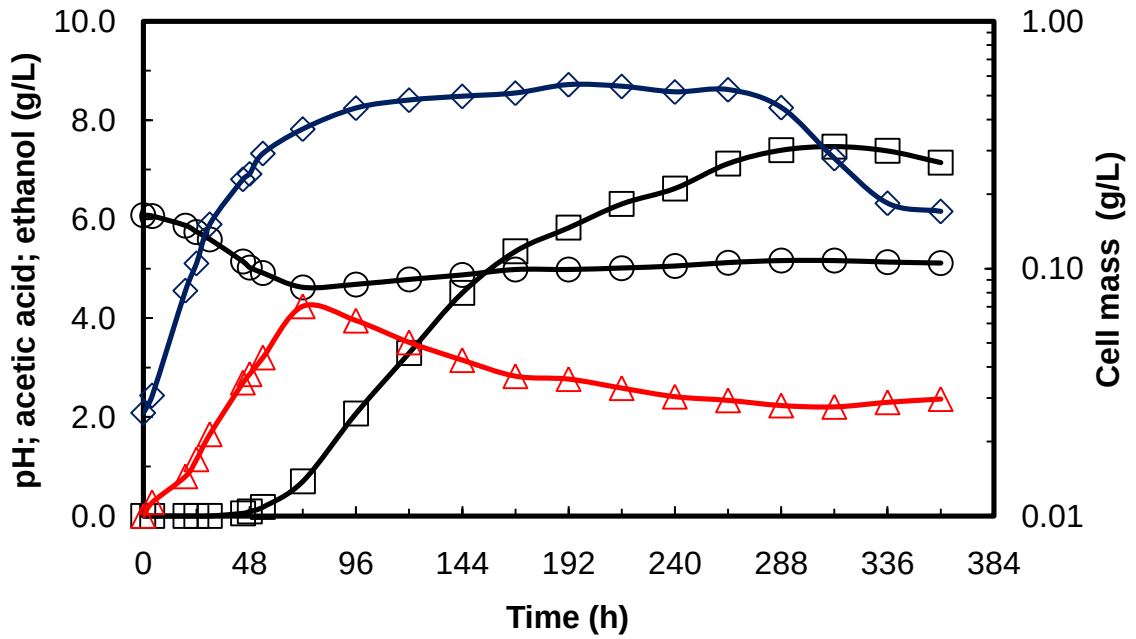

Figure 4.7 Growth, pH and product profiles using *Clostridium* strain P11 in 10 g/L corn steep liquor media: (◇) cell mass; (□) ethanol; (Δ) acetic acid; (O) pH.

The pH of the medium gradually decreased from 6.1 to 4.62 with simultaneous production of acetic acid (Figure 4.7). Acetic acid production increased during growth phase and reached a maximum concentration of 4.2 g/L after 72 h. The productivity of acetic acid in 10 g/L CSL medium was 6.6 mg/Lh. Ethanol production started after 48 h of fermentation. A shift from acidogenesis to solventogenesis was observed after 72 h, which is evident by the increase in the pH from 4.62 to 5.0. The rate of ethanol production was the highest between 72 and 144 h. The maximum ethanol concentration in the medium with 10 g/L CSL was 7.46 g/L after 312 h of fermentation with a corresponding productivity of 24 mg/Lh. However, ethanol concentration decreased to 7.14 g/L by the end of fermentation. After 312 h, no ethanol was produced. A slight decrease in ethanol concentration might be due to stripping by the flowing syngas. Ethanol concentration in the water trap was 17 g/L. The total amount of ethanol produced during fermentation was 8.6 g/L taking into account ethanol in water trap and in samples withdrawn from the fermentor. About 41% more ethanol was produced in the 10 g/L CSL medium compared to YE medium.

Figure 4.8 shows gas utilization by strain P11. The utilization of CO and H_2 were the highest during the growth and acidogenic phases. The conversion efficiencies of CO and H_2 during exponential phase were 19% and 21%, respectively. Utilization of CO continued till the end of stationary phase at 288 h, whereas H_2 consumption decreased drastically when cells were in the stationary phase with the shift to solventogenesis. In the solventogenic phase, CO was utilized as a carbon and energy source, producing ethanol as the major product and H_2 might be used as an electron donor because only about 2% of H_2 was consumed. CO_2 was produced during ethanol formation. Unlike in

the YE medium (Figure 4.3), almost no CO_2 was consumed in the 10 g/L CSL medium (Figure 4.8).

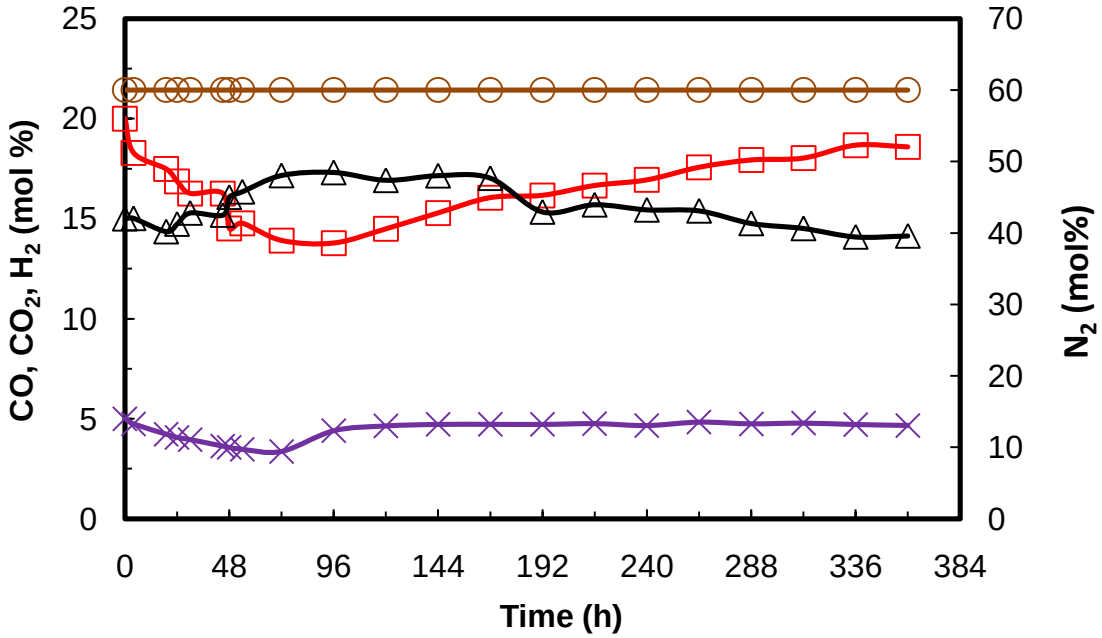

Figure 4.8 CO, CO_2, H_2 and pressure profiles using *Clostridium* strain P11 in 10 g/L corn steep liquor media: (□) CO; (Δ) CO_2; (×) H_2; (O) N_2.

The total number of moles of CO and H_2 consumed during 360 h of fermentation were 5.51 and 0.63 moles, respectively (Figure 4.9). Approximately, 36% and 69% of the total CO and H_2 consumed by strain P11 were utilized during the growth phase to make new cells and produce acetic acid. During the stationary phase, strain P11 utilized approximately 53% of the overall CO consumed and 21% of the total H_2 consumed for maintenance and ethanol production. H_2 consumed during the stationary phase might have been utilized by the cells to reduce acetate to ethanol (Equation 3.9). Also, H_2 oxidation by hydrogenases generates electrons or reducing equivalents necessary for ethanol production (Ragsdale 2004). The conversion efficiencies of CO and H_2 during 360 h of fermentation in the 10 g/L CSL medium were 15% and 7%, respectively (Table

4.5). Ethanol and acetic acid yields from CO were 57% and 9% of the theoretical values, respectively.

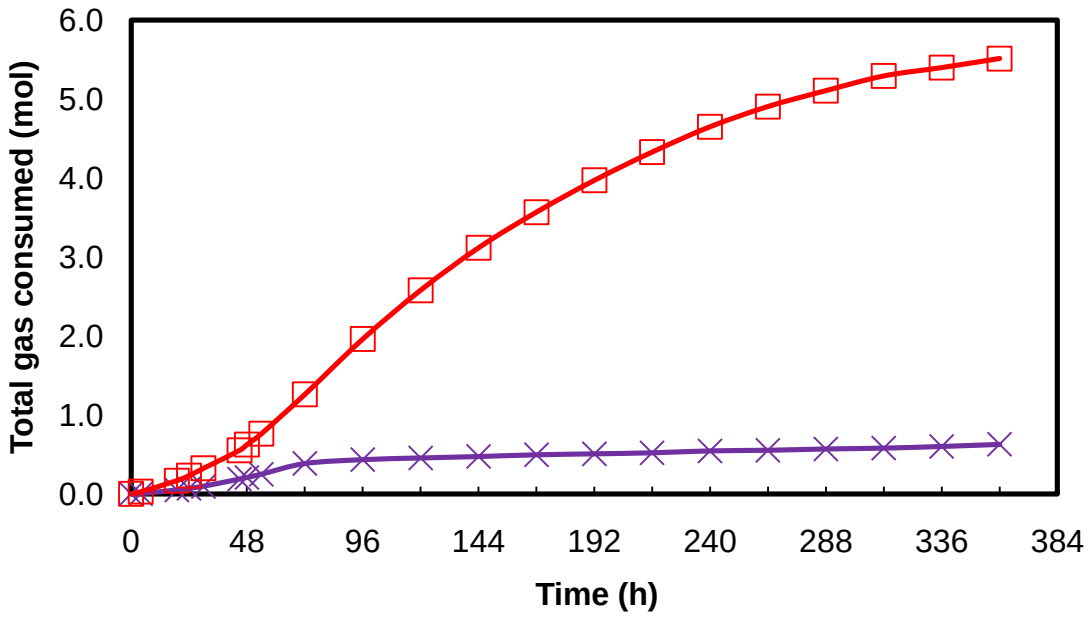

Figure 4.9 Total moles of CO and H_2 consumed by *Clostridium* strain P11 in 10 g/L corn steep liquor media: (□) CO and (×) H_2.

The redox potential (ORP) profile for strain P11 in 10 g/L CSL medium (Figure 4.10) was different compared to the YE medium (Figure 4.5). During the lag and beginning of growth phases in the 10 g/L CSL, the ORP values sharply dropped from +89 to -83 mV SHE. The ORP then increased in the growth phase to about -42 mV. However, it continued to drop during the stationary phase. In the YE medium, the ORP value increased during the stationary phase (Figure 4.5). The ORP increased with the onset of cell death in both media. Ethanol production was noticed when the ORP values were between -45 to -140 mV SHE.

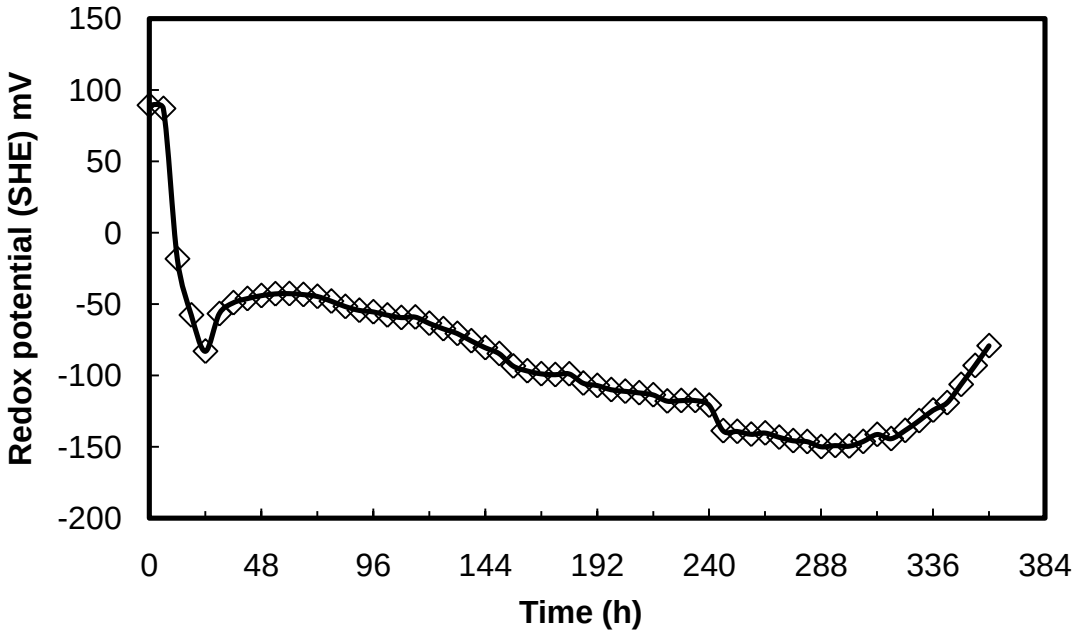

Figure 4.10 Redox potential profile of *Clostridium* strain P11 in 10 g/L corn steep liquor medium.

Figure 4.11 shows the concentration profiles of trace metals and minerals during syngas fermentation in the 10 g/L CSL. Approximately, 40% of the initial Molybdenum (Mo) (Figure 4.11b) and 6% of Tungsten (W) (Figure 4.11a) supplied were consumed during cell growth phase. There was no consumption of Ni, Se, Co, Zn and Fe in 10 g/L CSL medium. The concentration profiles of all other trace metals and elements during the fermentation in 10 g/L CSL medium are available in Appendix A.

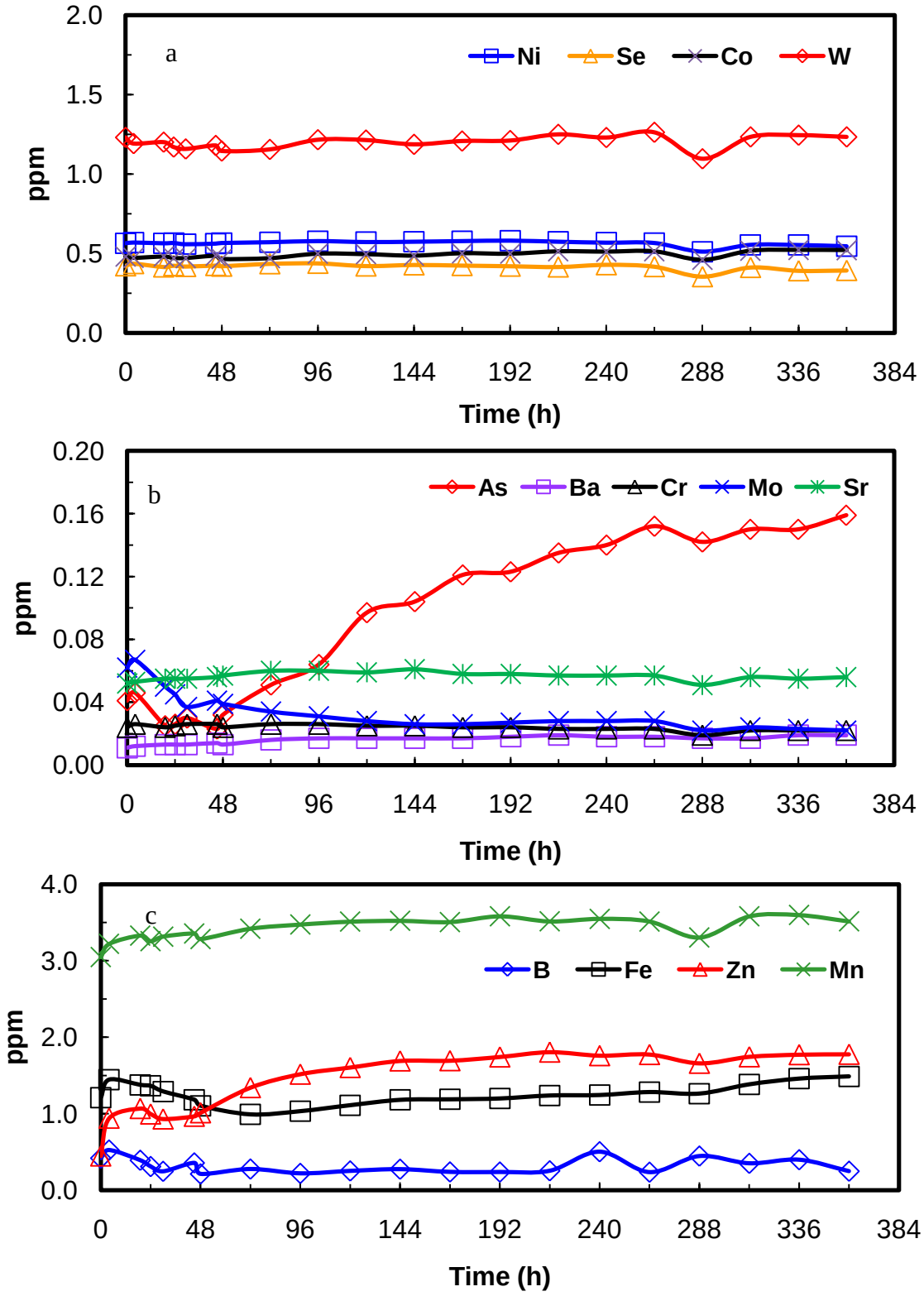

Figure 4.11 Trace metals and minerals profiles during syngas fermentation by strain P11 in 10 g/L corn steep liquor medium.

4.3.3 Fermentation in 20 g/L corn steep liquor medium

The growth and product profiles of strain P11 in the 20 g/L CSL medium are shown in Figure 4.12. Cells were in lag phase for 4 h, after which, their concentration increased exponentially from 0.045 g/L to 0.38 g/L in the first 48 h of cultivation. The maximum specific growth rate of P11 in this medium was 0.075 h^{-1}, which is 36% and 23% higher than in YE and 10 g/L CSL media, respectively (Table 4.5). Similar to the 10 g/L CSL medium, the exponential phase was followed by a deceleration phase from 48 h to 144 h. The cell concentration was about 0.69 g/L during the stationary phase. It then decreased to 0.46 g/L by the end of fermentation. The maximum cell concentration in the 20 g/L CSL medium was 0.74 g/L, which was obtained at 240 h. About 35% to 40% more cells were produced in the 20 g/L CSL medium compared to 1 g/L YE and 10 g/L CSL media. This was due to the presence of more nutrients in the 20 g/L CSL medium.

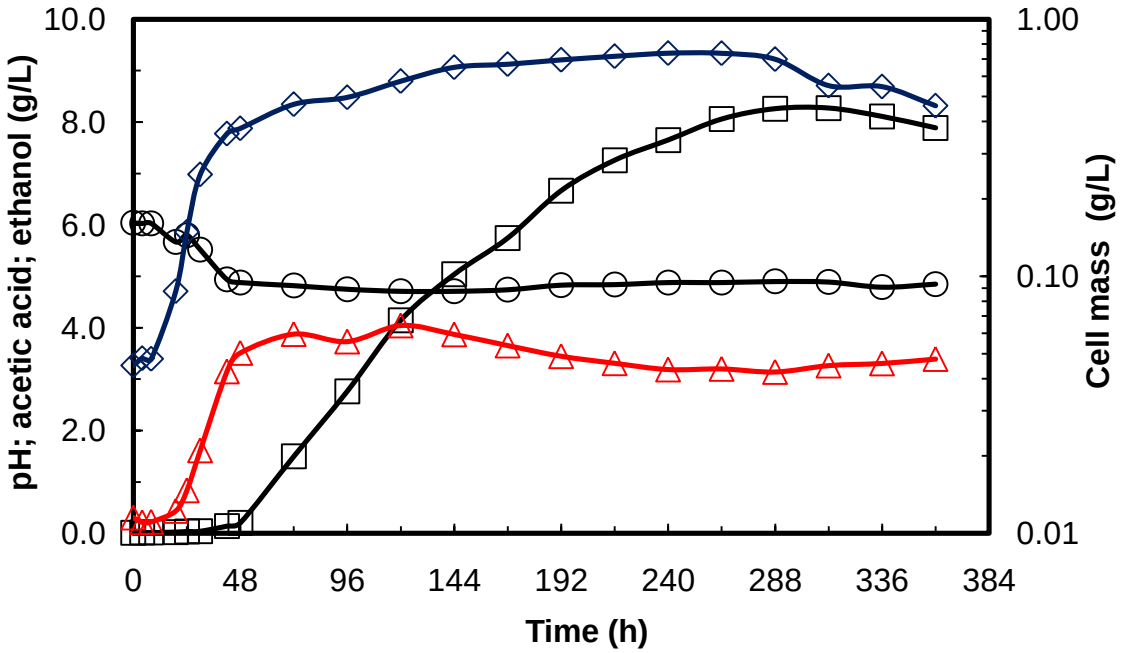

Figure 4.12 Growth, pH and product profiles using *Clostridium* strain P11 in 20 g/L corn steep liquor: (◇) cell mass; (□) ethanol; (Δ) acetic acid; (O) pH.

The pH of the fermentation broth decreased during growth phase from 6.1 to 4.8 due to acetic acid formation (Figure 4.12). The pH was around 4.8 for the remaining fermentation. Unlike in either 1 g/L YE or 10 g/L CSL media, no pH increase was noticed in the 20 g/L CSL medium. This was due to lower consumption of acetic acid in the 20 g/L CSL medium. The maximum amount of acetic acid produced during the growth and deceleration phases was 4 g/L with a productivity of 9.4 mg/Lh. A shift from acidogensis to solventogenesis was observed after about 48 h, at which ethanol production started. Only about 1 g/L of acetic acid was consumed during the stationary phase. Ethanol concentration increased from 0.2 to 8.27 g/L between 48 to 312 h. The productivity of ethanol was 27 mg/Lh at 312 h. Then, ethanol concentration in the fermentor decreased, possibly due to stripping by the flowing syngas as was also seen in the 10 g/L CSL medium (Figure 4.7). The concentration of ethanol in the water trap was 18 g/L. When ethanol in the water trap and withdrawn samples is included in the calculation of the total ethanol produced, the concentration of ethanol in the fermentor is equal to 9.6 g/L. About 57% and 12% more ethanol were produced in the 20 g/L CSL medium compared to the 1 g/L YE and 10 g/L CSL media, respectively.

Both CO and H_2 utilization by strain P11was detected during exponential and early stationary phase (Figure 4.13). CO consumption continued during the stationary phase, while H_2 consumption almost ceased after 96 h. CO utilization in the 10 g/L and 20 g/L CSL media was 36% higher than in 1 g/L YE medium. However, the utilization of H_2 in 10 g/L CSL medium was 38% and 31% higher compared to 1 g/L YE and 20 g/L CSL media. The average consumption of CO and H_2 by strain P11 in the first 48 h of fermentation were 20% and 25%, respectively, of the total supplied gas.

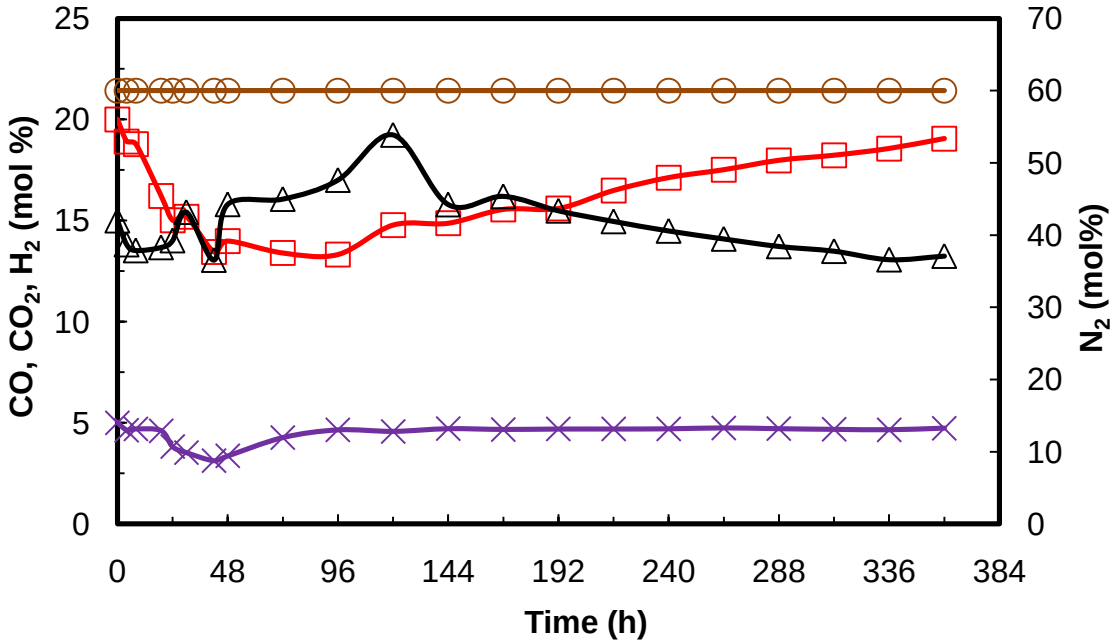

Figure 4.13 CO, CO_2, H_2 and pressure profiles using *Clostridium* strain P11 in 20 g/L corn steep liquor medium: (□) CO; (Δ) CO_2; (×) H_2; (O) N_2.

The total number of moles of CO and H_2 consumed by strain P11 during the fermentation were 5.80 and 0.48, respectively (Figure 4.14). About 28% of the total CO and 56% of total H_2 consumed by the cells were utilized during the growth phase for cell growth and acetic acid formation. Cells in the stationary phase utilized approximately 68% of the total CO and 32% of the total H_2 consumed for their maintenance and ethanol production. Ethanol and acetic acid yields based on CO consumption were 60% and 12% of the theoretical values, respectively (Table 4.5). The overall conversion efficiencies of CO and H_2 in the 20 g/L CSL medium were 15% and 5%, respectively.

Figure 4.15 shows the redox potential profile for strain P11 in 20 g/L CSL medium. There was an increase in ORP of the medium in the lag phase. The ORP then dropped sharply from -22 to about -120 mV SHE during the exponential growth phase. The ORP in the medium remained around -110 mV SHE during the stationary phase. It then sharply increased during cell death phase. Acetic acid production was noticed

between -22 and -110 mV SHE, while ethanol formation was between about -35 to -130 mV SHE.

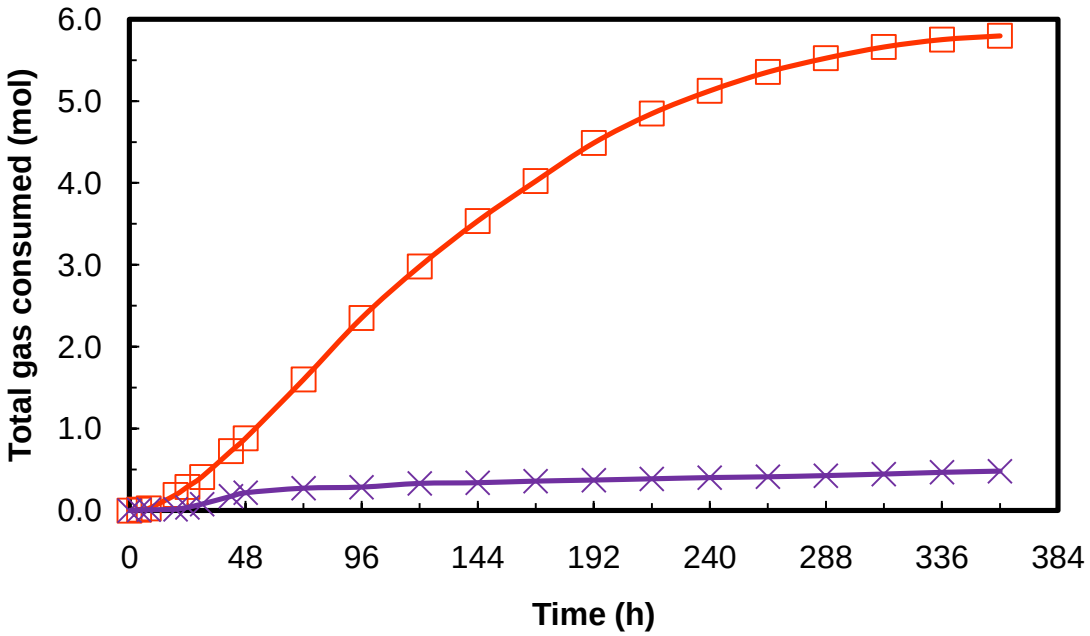

Figure 4.14 Total moles of CO and H_2 consumed by *Clostridium* strain P11 in 20 g/L corn steep liquor medium: (□) CO and (×) H_2.

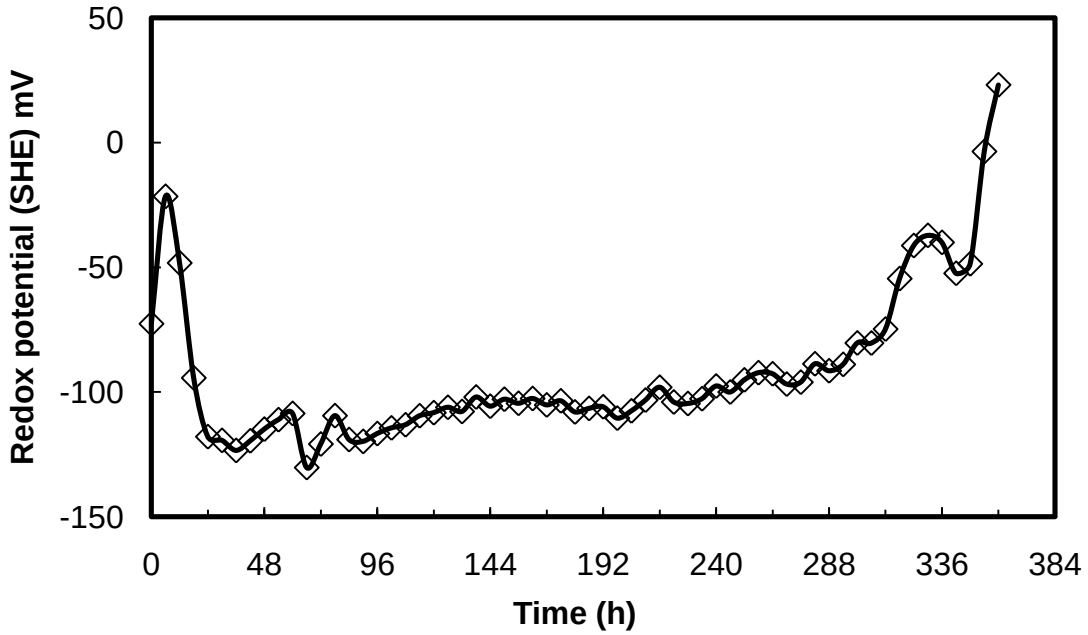

Figure 4.15 Redox potential profile of *Clostridium* strain P11 in 20 g/L corn steep liquor medium.

Figure 4.16 shows the concentration profiles of trace metals and minerals during syngas fermentation in 20 g/L CSL medium. The concentrations of most of the trace metals and minerals did not change during the fermentation process. However, the concentrations of tungsten (W), arsenic (As), molybdenum (Mo) changed during the fermentation. The concentration of W increased from about 1.2 ppm to 2.2 ppm at the late stage of the fermentation when cells concentration dropped (Figure 4.16a), while approximately 60% of Mo supplied was consumed during the growth phase (Figure 4.15b). There were sudden spikes in the concentration of few elements at 124 h. The reasons for these spikes are not known. In addition, the trace metals and minerals available in the 20 g/L CSL medium were not depleted from the fermentation medium. This shows that there were enough trace metals and minerals in the 20 g/L CSL medium. The concentration profiles of other trace metals and elements as well as total nitrogen and carbon during fermentation in the 20 g/L CSL medium are available in Appendix A.

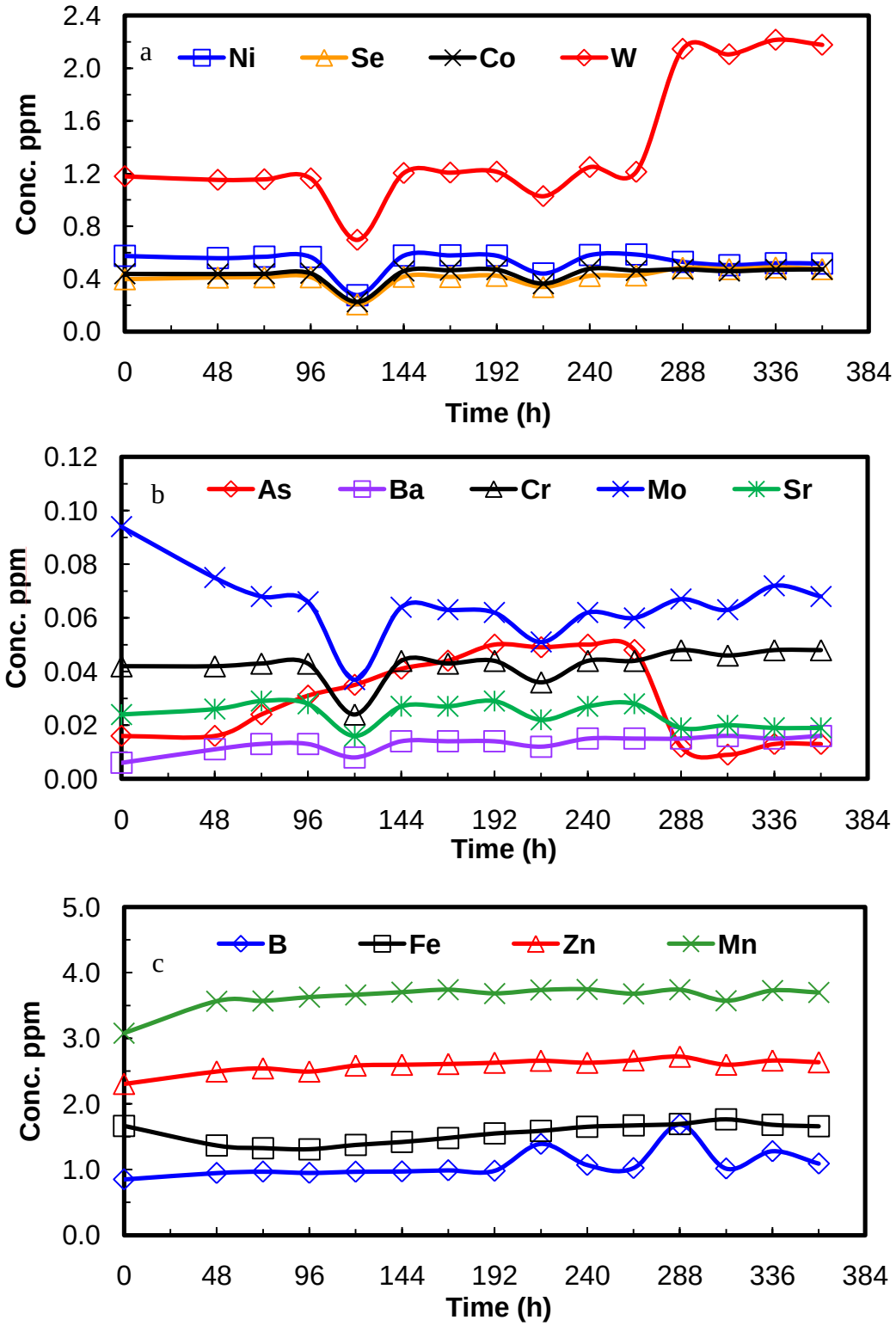

Figure 4.16 Trace metals and minerals profiles during syngas fermentation by strain P11 in 20 g/L corn steep liquor medium.

The results demonstrated that CSL can be used as the major nutrient source in syngas fermentation. Significant increase in ethanol formation by strain P11 of 57% and 41% were observed in the 10 g/L and 20 g/L CSL media, respectively, compared to standard 1 g/L YE medium (Table 4.5). Generally, similar fermentation profiles were noticed in all three media. There was a short lag phase followed by an exponential phase. The cell concentrations in all three media increased exponentially during the first 48 h of fermentation. The specific growth rates of strain P11 were 0.055, 0.061 and 0.075 h^{-1} in the 1 g/L YE, 10 g/L CSL and 20 g/L CSL media, respectively (Table 4.5). The specific growth rates of *Clostridium ljungdahlii* (Phillips et al. 1994), and *Clostridium* strain P11 (Frankman 2009) in yeast extract medium were reported as 0.06 h^{-1} and 0.0491 h^{-1}, respectively. These specific growth rates are lower than those with strain P11 in the 20 g/L CSL medium. The maximum cell concentration in the 20 g/L CSL medium was higher than the other two media due to the presence of more nutrients in the 20 g/L CSL medium.

CSL is rich in nutrients such as water soluble vitamins, amino acids, minerals, trace metals (Liggett and Koffler 1948) and small fraction of carbohydrates (Amartey and Jeffries 1994). The presence of more carbohydrates and other nutrients in the 20 g/L CSL medium could have contributed to the higher performance of strain P11 in this medium compared to standard 1 g/L YE medium. The initial total concentrations of sugars in the 10 g/L and 20 g/L CSL media were very small, 0.52 and 1.05 g/L, respectively. The utilization of sugars during fermentation was measured at 0, 120, 240 and 360 h. The initial glucose concentrations in the 10 g/L and 20 g/L CSL media were 0.12 g/L and 0.32 g/L, respectively. Only 10% and 76% of the total initial amounts of glucose in

10 g/L CSL and 20 g/L CSL media were consumed, respectively (Figures 4.17 and 4.18). This indicates that strain P11 consumed more glucose in 20 g/L CSL medium than in the10 g/L CSL medium. Strain P11 consumed about 40% of the total sugars present in 20 g/L CSL medium; whereas, only 2% of the total sugars were consumed in 10 g/L CSL medium. The maximum possible amount of ethanol production assuming 100% conversion efficiency of glucose to ethanol in the 10 g/L and 20 g/L CSL media are 0.006 g/L and 0.16 g/L, respectively. These amounts of ethanol from the sugars present in CSL are negligible in comparison to ethanol produced from syngas. Therefore, it is clear that all the ethanol produced during syngas fermentation in 10 g/L and 20 g/L CSL media is due to syngas conversion of syngas. More details on the sugars consumption during syngas fermentation is shown in Appendix B.

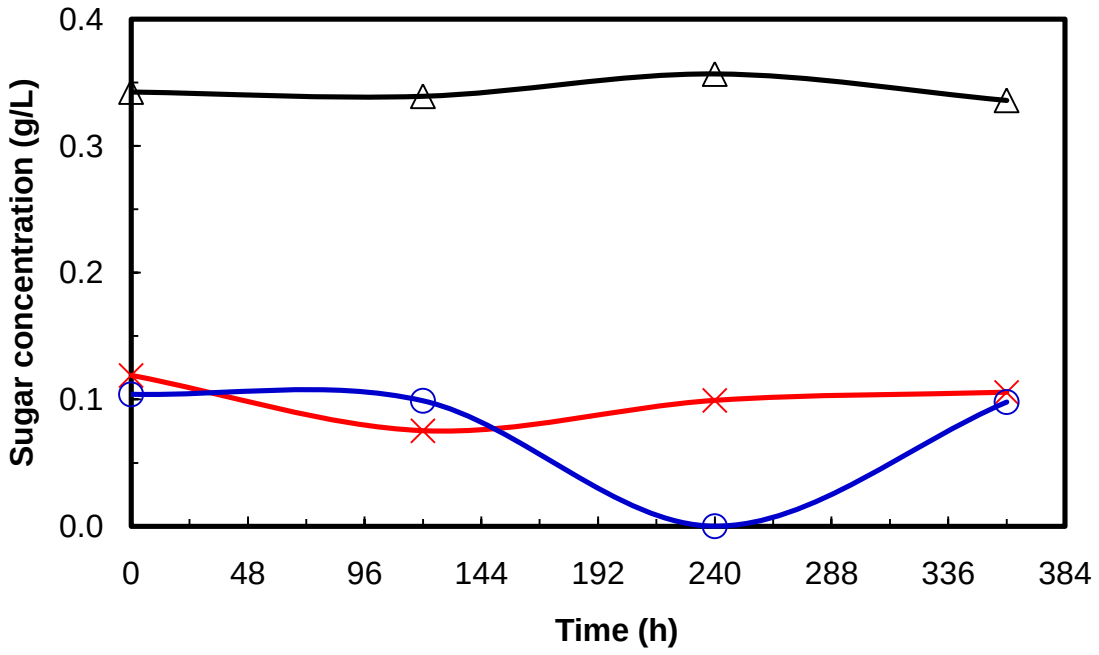

Figure 4.17: Sugars utilization profiles of strain P11 in 10 g/L corn steep liquor medium: (×) Glucose; (O) Mannose; (Δ) Cellobiose.

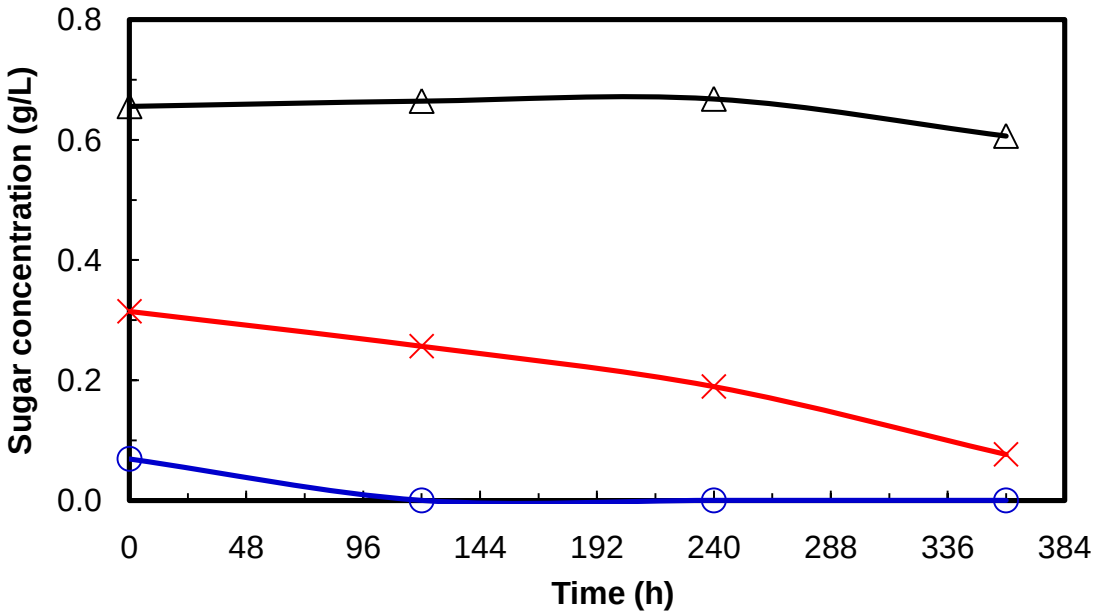

Figure 4.18: Sugars utilization profiles of strain P11 in 20 g/L corn steep liquor medium: (×) Glucose; (O) Mannose; (Δ) Cellobiose.

Product formation in all media with *Clostridium* strain P11 was seen during the acidogenic and solventogenic phases similar to other acetogens (Diekert and Wohlfarth 1994). Strain P11 utilizes the reductive acetyl-CoA pathway for cell growth and product formation. Cells produce ATP during the reduction of acetyl-CoA to acetate in the acetogenic phase. Acetic acid formation by strain P11 was growth related, which resulted in a decrease in the pH of the fermentation medium (Figures 4.2, 4.7 and 4.12). In addition, acetic acid consumption was observed in all media used. However, the consumption of acetic acid was the highest in the 1 g/L YE medium (Table 4.5). It is hypothesized that acetic acid in all media was reduced to ethanol during the solventogenic phase (Equation 3.9).

The switch to solventogenesis and production of ethanol was detected during deceleration growth and stationary phases when the pH was around 4.6 (Figures 4.2, 4.7

and 4.11). This indicates that ethanol formation in strain P11 is non-growth associated and has also been reported with other microorganisms such as *Clostridium carboxidivorans* $P7^T$ (Ahmed and Lewis 2007; Rajagopalan et al. 2002), *Clostridium ljungdahlii* (Hurst and Lewis 2010), *Clostridium autoethanogenum* (Cotter et al. 2009). The rate of ethanol production in all media was high in the early stationary phase and decreased with time especially with the decline in cell mass concentration. After 360 h of fermentation, ethanol concentrations in 1 g/L YE, 10 g/L CSL and 20 g/L CSL media were 6.1, 8.6 and 9.6 g/L, respectively (Table 4.5). About 4.8 g/L of ethanol was reported with P11 and syngas composition of 20% CO, 30% H_2 and 50% CO_2 (Frankman 2009).

From gas consumption profiles during syngas fermentation, it is clear that strain P11 utilized more CO than H_2 for growth and product formation (Figures 4.3, 4.8 and 4.13). Moreover, small amounts of CO_2 were consumed in the fermentation media with a net CO_2 production especially in both CSL media (Figures 4.8 and 4.13). CO_2 consumption by the cells could be for ethanol or acetic acid production according to Equations 3.2 and 3.4. Based on Gibbs free energy, the formation of acetic acid is more favorable than ethanol from CO_2 (Table 3.1). This is also supported by the current results because CO_2 utilization by strain P11 was only noticed in the growth phase in which acetic acid was produced. The rate of CO consumption was high in the deceleration and stationary phases. This implies that cells consumed CO to produce acetic acid, ethanol and CO_2 according to Equations 3.1 and 3.3. Similar gas consumption profiles were observed by Cotter et al. (2009) during syngas fermentation by *C. ljungdahlii*. The H_2 consumption in the present study was high during exponential growth phase in all

fermentation media. This indicates that most of the H_2 was utilized for acetic acid production.

In acetyl-CoA pathway, both H_2 and CO serve as an energy and electron source for cell growth and product formation. Significant amounts of carbon from CO can be converted to cell material and ethanol if H_2 is utilized as an electron source. The depletion of H_2 in the fermentation medium would reduce the amount of carbon available for ethanol production because a fraction of CO would be utilized for generating reducing equivalents required (Ahmed and Lewis 2007). Therefore, increasing the concentration of H_2 in the gas phase would increase the contribution of CO to ethanol production.

Moreover, it was reported that increased H_2 partial pressure improved alcohol yields from glucose by *C. acetobutylicum* (Yerushalmi et al. 1985). However, in the current research, syngas was fed continuously to supply strain P11 with enough CO and H_2 to convert these gases to ethanol. In addition, high concentrations of CO were found to inhibit hydrogenase enzyme and thus reducing the uptake of H_2 (Kim et al. 1984). This could explain the low consumption of H_2 during the stationary phase with strain P11.

From syngas composition profile during fermentation (Figure 4.3, 4.8 and 4.13), it can be seen that H_2 uptake was low when the percentage of CO in the head space was generally above 14% (volume %). A similar trend in reduction of H_2 consumption was observed when *E. limosum* was grown on CO and H_2 (Genthner and Bryant 1982). The conversion efficiencies of CO and H_2 in the present study were over 14% and 6%, respectively (Table 4.5). The low conversion efficiency was due to the continuous supply of syngas at a rate that strain P11 could not utilize. To enhance the conversion efficiencies of CO and H_2, lower syngas flow rates and/or higher initial cell

concentrations in the fermentor should be used. The ethanol yields from CO in all media were above 53% of the theoretical values.

4.5 Conclusions

Syngas fermentation by *Clostridium* strain P11 was successfully demonstrated using three different media formulations in a 7.5-L bench-top fermentor operated at 143 kPa (absolute) and 37°C. Results showed that corn steep liquor (CSL) can be used as the primary nutrient for syngas fermentation and can totally replace expensive yeast extract (YE). Ethanol productivities in the 10 g/L and 20 g/L CSL media were 41% and 59%, respectively, higher than in the 1 g/L YE medium. This was due to the sustained higher cell densities in the CSL media compared to the YE medium. The medium with 20 g/L CSL produced the highest amount of ethanol (9.6 g/L) compared to the 1 g/LYE medium (6.1 g/L). Little difference in ethanol production was measured in media with 10 g/L CSL (8.6 g/L) and 20 g/L CSL (9.6 g/L). However, the 20 g/L CSL medium is 28% less expensive than the 10 g/L CSL medium as shown in Table 3.5. These results are important when considering a fermentation medium for large scale ethanol production.

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CHAPTER V

FUTURE WORK AND RECOMMENDATIONS

Based on the results obtained in this work concerning the evaluation of corn steep liquor as the main medium component for ethanol production from syngas using *Clostridium* strain P11, the following recommendations are made for future studies:

- Media optimization for strain P11 using statistical experimental design methods such as Response Surface Methodology (RSM) and Plackett–Burman with an objective to further reduce the media cost by eliminating unnecessary nutrients such as certain vitamins and/or minerals. The ICP analysis of the samples taken during syngas fermentation in the 7.5-L fermentor showed that there were no deficiencies of any metals or elements in the three media used. However, the concentrations of the metals and trace elements in the three media were different (Table 4.4). In addition, the concentrations of several minerals and trace metals (Mo, B, Se, Co, Ni, W, Fe, Mn, and K) were within one order of magnitude of each other in the three fermentations used. The highest ethanol concentration was achieved in the 20 g/L CSL medium, which was 57% higher than the 1 g/L YE medium. Therefore, the composition of amino acids and vitamins present in CSL should also be determined, which could explain why the 20 g/L CSL medium

outperformed the 1 g/L YE medium. This study will allow further optimization of the nutrients used in syngas fermentation media.

- Investigate the effect of partial pressures of CO, H_2 and CO_2 inside the fermentor on the rate of their uptake by strain P11 and on ethanol production rates during syngas fermentation. It is important to explore methods that would allow a sustained and simultaneous utilization of CO, H_2 and CO_2 with high ethanol yields during extended operations of batch and continuous fermentations.
  In addition, syngas fermentation in YE and CSL media using producer gas made from gasifying switchgrass and other biomass feedstocks should be investigated. Research on the effects of CO, H_2 and CO_2 on key enzymes involved in the metabolic pathway for ethanol production is ongoing by members of the syngas fermentation group at Oklahoma State University (OSU) and Brigham Young University (BYU). The results of the enzyme study will help in process development by selecting the range of gas compositions that would provide the highest ethanol production.
- The conversion efficiencies of syngas components (CO and H_2) and ethanol yields were low in the fermentor due to mass transfer limitations, low solubility of CO and H_2 in the fermentation medium and low cells concentration. The following design equation expresses the overall mass transfer coefficient, K_La as a function of agitator power-per-volume ratio (P/V) and the superficial gas velocity (u_g) in a CSTR.

$$K_La = K\left(\frac{P}{V}\right)^{\alpha}\left(u_g\right)^{\beta} \quad (5.1)$$

Where, K_L is the mass transfer coefficient, a is the gas/liquid interface area per liquid volume, α and β are constants that depend on reactor geometry, impeller type and design, and continuous phase properties. From Equation 5.1, it can be determined that under mass transfer limitations, increasing the impeller speed would increase (P/V) and K_La. The increase in K_La would increase the rate of mass transfer from the gas to the liquid phase. The increase in K_La is expected to increase the reactor productivity. However, on an industrial scale high P/V ratio would make the process expensive. Moreover, increasing the gas flow rates would increase superficial gas velocity and hence K_La. But for reactions involving sparingly insoluble gases at high gas flow rates could result in low gas conversion efficiencies. Also, gas recycle at desired ratios might improve the gas conversion efficiency (Bredwell et al. 1999). Therefore it is necessary to determine the optimum gas flow rate, gas composition, agitation speed and gas and cell recycle ratios to the fermentor in order to enhance ethanol production. New reactor designs such as hollow fiber membrane reactors (Tsai et al. 2009a; Tsai et al. 2009b) should also be explored to overcome some of the mass transfer limitations and low cell density in the CSTR.

- Production of butanol (below 1 g/L) by strain P11 was measured in the yeast extract and corn steep liquor media in 250-mL serum bottles after 600 h of syngas fermentation (Table 3.3). Butanol has many advantages as a fuel compared to ethanol. It has higher energy density than ethanol and is compatible with existing pipeline fuel distribution systems and car engines. Methods to enhance butanol production from syngas should be explored. This includes studying the enzymes

involved in butanol production through the metabolic pathway of strain P11 and the factors that would enhance their activities. Some of this work is ongoing at OSU, Stillwater and the University of Oklahoma, Norman.

5.1 References

Bredwell MD, Srivastava P, Worden RM. 1999. Reactor Design Issues for Synthesis-Gas Fermentations. Biotechnology Progress 15(5):834-844.

Tsai S-P, Datta R, basu R, Yoon S-H; 2009a. Horizantal array bioreactor for conversion of synthesis gas components to liquid products. USPTO-20090215142.

Tsai S-P, Datta R, basu R, Yoon S-H; 2009b. Stacked array bioreactors for conversion of synags components to liquid products. USPTO-20090215153.

APPENDIX A

The concentration profiles of trace metals and elements during syngas fermentation

A.1 Yeast extract medium (1 g/L)

The concentration profiles of several trace metals and elements were shown in figure 4.6. The profiles of other metals, minerals, total nitrogen and carbon during syngas fermentation in 1 g/L YE medium are shown in Figure A1 and A2.

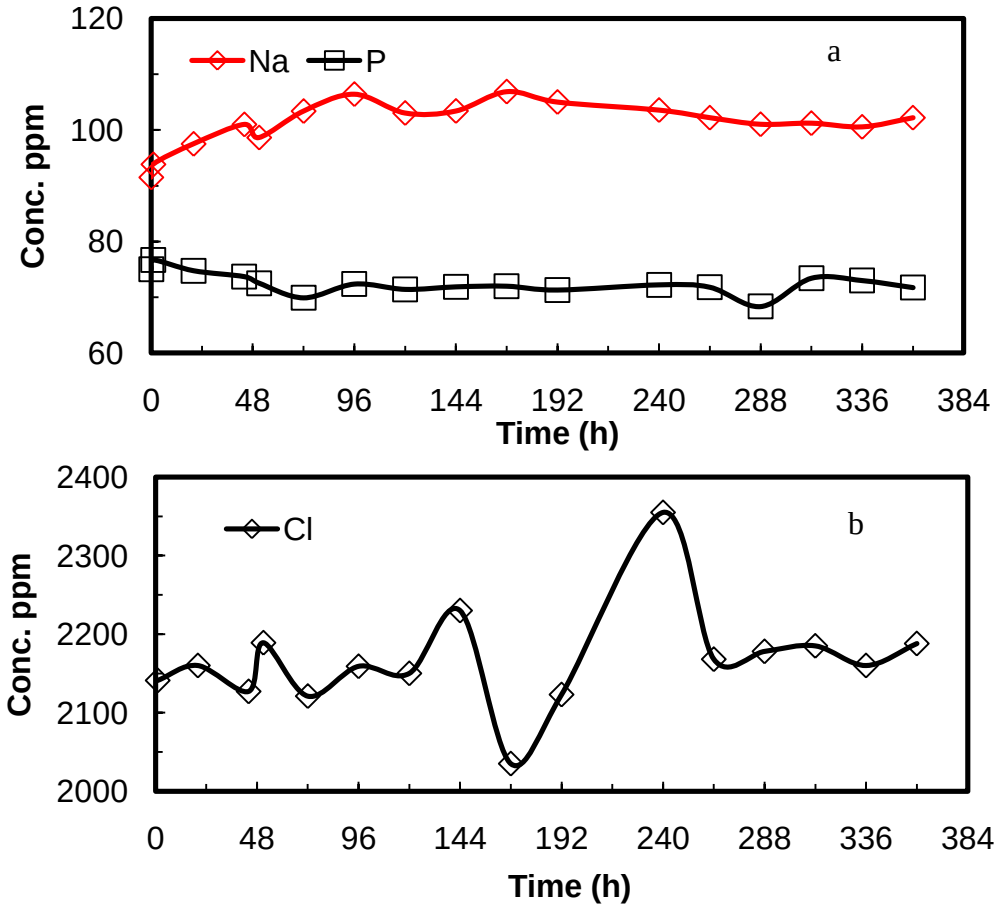

Figure A.1 Trace metals and minerals profiles during syngas fermentation by strain P11 in 1 g/L yeast extract medium.

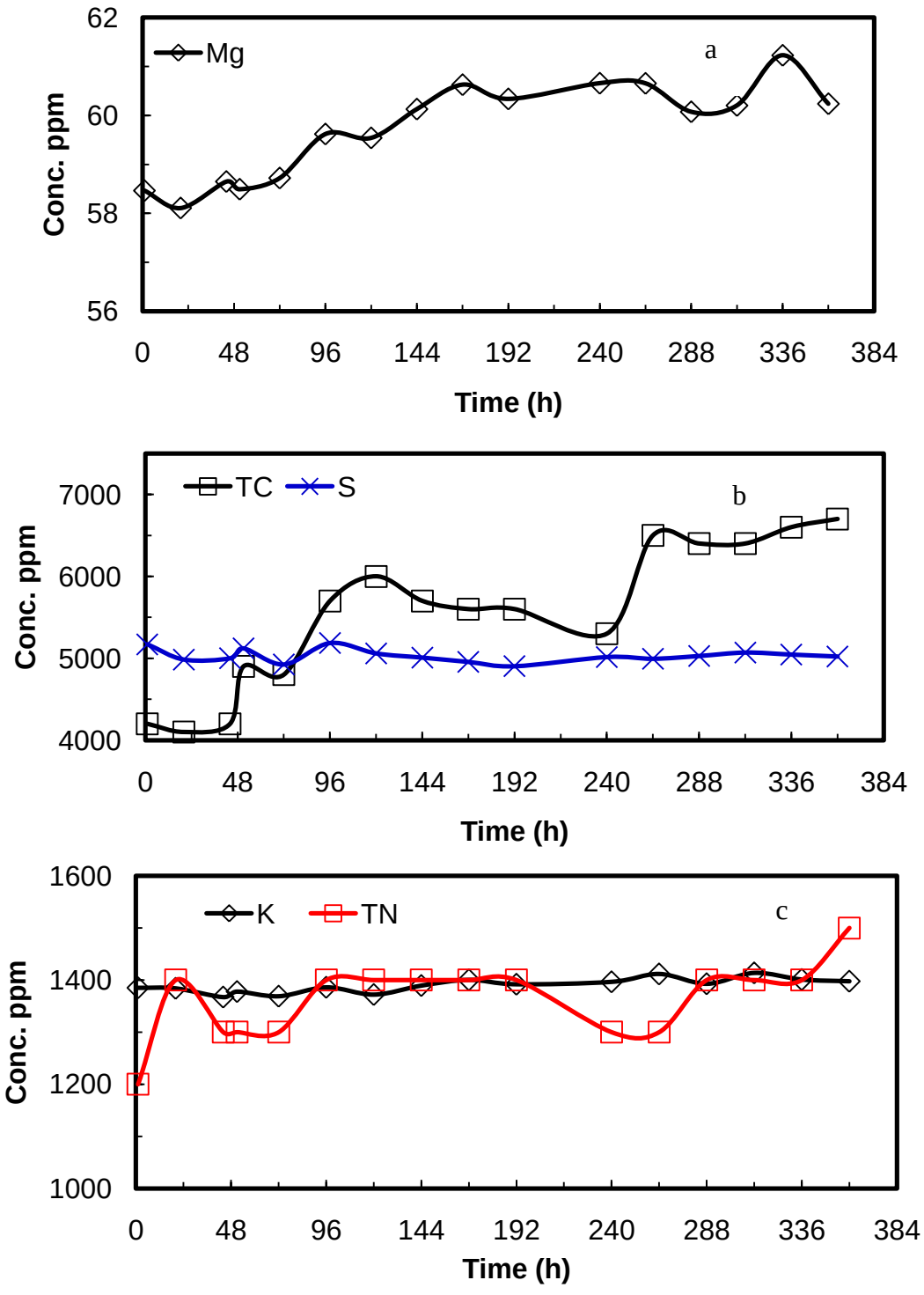

Figure A.2 Trace metals and minerals profiles during syngas fermentation by strain P11 in 1 g/L yeast extract medium.

Table A.1 Accuracy of analysis of trace metals and minerals on ICP for 1 g/L yeast extract medium for calibration standards and known samples.

Element	Calibration standard (ppm)	True Value (ppm)	% error [a]	Known Concentration sample (ppm)	True Value (ppm)	% error [a]
As	1.00	1.02	2.40	0.18	0.17	-7.78
Ba	1.00	1.01	1.00	0.32	0.30	-3.80
Co	1.00	1.01	1.20	0.38	0.36	-5.32
Cr	1.00	1.01	0.80	0.40	0.38	-5.24
Mo	1.00	1.01	0.70	0.40	0.21	-2.77
Ni	1.00	1.01	-0.80	0.65	0.65	0.15
Se	1.00	1.02	2.20	0.79	0.75	-5.32
Sr	1.00	1.01	0.60	0.17	0.17	0.00
W	1.00	0.94	-6.00	-	-	-
Cl	120.00	117.00	-2.50	-	-	-
Na	10.00	11.20	12.04	50.00	54.26	8.51
Ca	10.00	10.19	1.90	50.00	53.64	7.27
Mg	10.00	10.28	2.83	50.00	52.32	4.65
K	10.00	10.75	7.46	50.00	53.19	6.38
S	10.00	10.20	1.96	50.00	51.44	2.88
B	0.200	0.21	3.50	0.50	0.51	2.20
	-	-	-	0.92	0.91	-1.09
P	1.00	1.05	4.70	0.50	0.55	10.70
Fe	1.00	1.01	0.60	0.50	0.55	9.90
	-	-	-	1.44	1.40	-2.64
Zn	1.00	1.04	3.00	0.50	0.05	31.70
	-	-	-	0.56	0.58	0.35
Cu	1.00	1.03	3.00	0.50	0.52	3.60
	-	-	-	0.42	0.40	-3.81
Mn	1.00	1.04	3.00	0.50	0.51	2.70
	-	-	-	1.54	1.51	-2.20
Al	0.20	0.20	0.00	0.32	0.34	7.94

a- percentage relative error

A.2 Corn steep liquor medium (10 g/L)

The concentration profiles of other trace metals and elements as well as total nitrogen and carbon during the fermentation in 10 g/L CSL medium are shown below in figures A.3 and A.4.

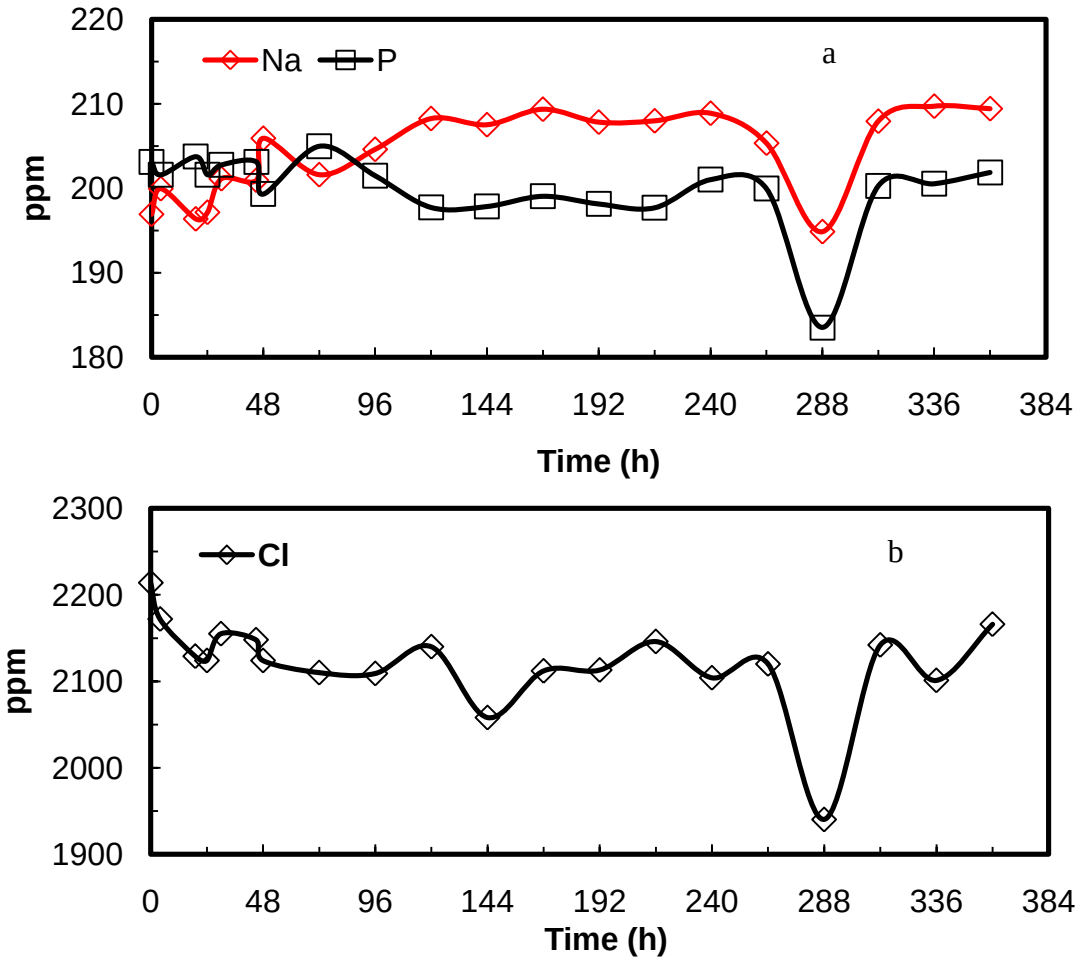

Figure A.3 Trace metals and minerals profiles during syngas fermentation by strain P11 in 10 g/L corn steep liquor medium.

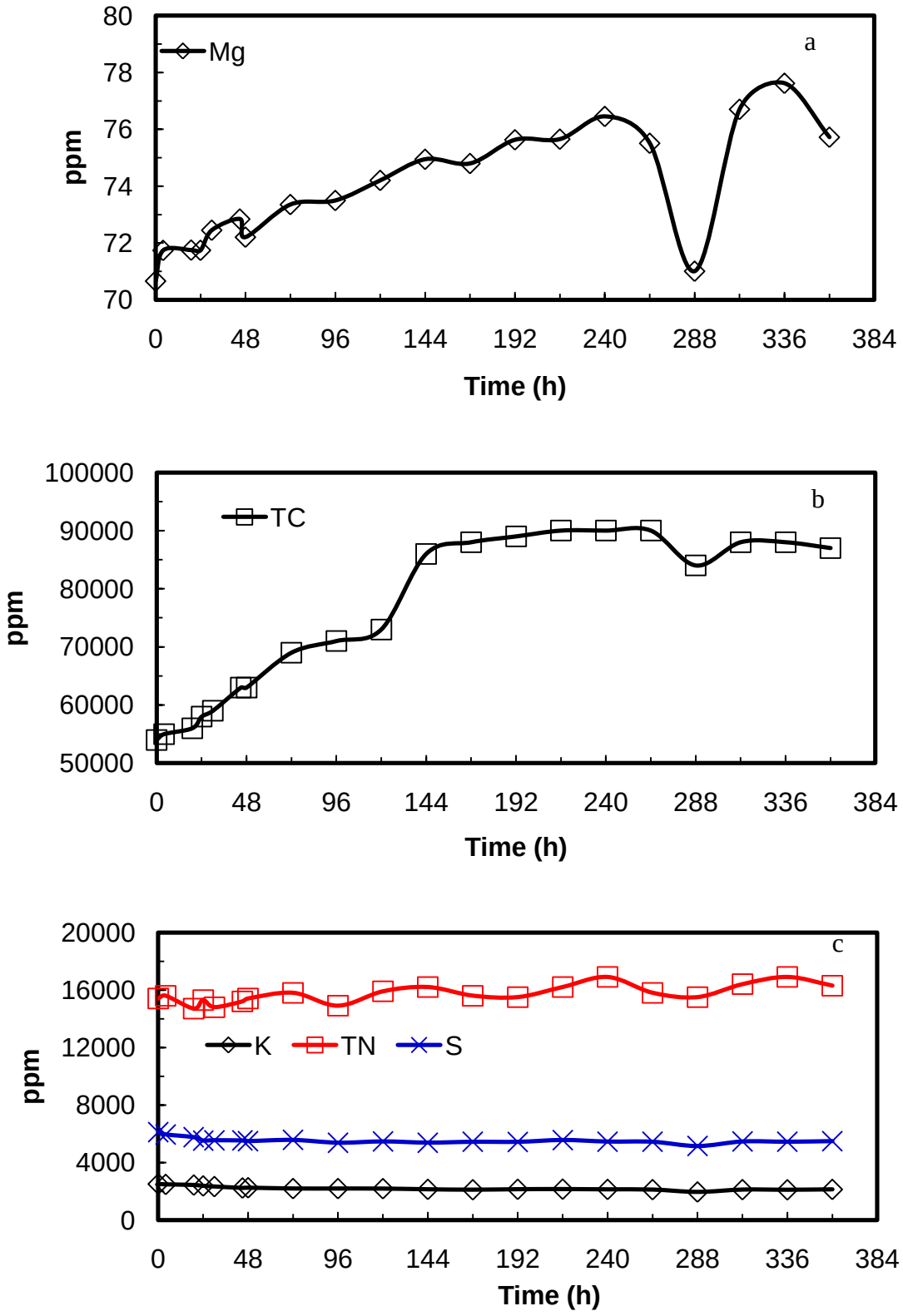

Figure A.4 Trace metals and minerals profiles during syngas fermentation by strain P11 in 10 g/L corn steep liquor medium.

Table A.2 Accuracy of analysis of trace metals and minerals on ICP for 10 g/L corn steep liquor medium for calibration standards and known samples.

Element	Calibration Standard (ppm)	True Value (ppm)	% error[a]	Known Concentration sample (ppm)	True Value (ppm)	% error[a]
As	0.10	0.098	-2.00	0.17	0.16	-6.21
	0.01	0.017	70.00	-	-	-
Ba	0.10	0.099	-1.00	0.31	0.30	-4.41
	0.01	0.010	0.00	-	-	-
Co	0.10	0.099	-1.00	0.37	0.35	-4.30
	0.01	0.009	-10.00	-	-	-
Cr	0.10	0.099	-1.00	0.40	0.38	-5.23
	0.01	0.014	40.00	-	-	-
Mo	0.10	0.100	0.00	0.22	0.21	-5.80
	0.01	0.012	20.00	-	-	-
Ni	0.10	0.100	0.00	0.65	0.65	-0.61
	0.01	0.010	0.00	-	-	-
Se	0.10	0.106	6.00	0.75	0.74	-1.321
	0.01	0.013	30.00	-	-	-
Sr	0.10	0.099	-1.00	0.17	0.16	-2.87
	0.01	0.011	10.00	-	-	-
W	0.10	0.102	2.00	-	-	-
	0.01	0.013	30.00	-	-	-
Cl	-	-	-	117.79	120.00	1.87
Na	100.00	102.564	2.56	50.00	54.93	9.86
	100.00	101.297	1.29	50.00	54.81	9.62
Ca	100.00	103.847	3.84	50.00	54.33	8.68
	100.00	100.065	0.06	50.00	55.50	11.04
Mg	100.00	102.735	2.73	50.00	54.63	9.27
	100.00	98.917	-1.08	50.00	54.15	8.31
K	100.00	102.388	2.38	50.00	54.46	8.92
	100.00	100.335	0.33	50.00	54.06	8.12
S	100.00	102.170	2.17	50.00	54.02	8.04
	100.00	99.542	-0.45	50.00	54.07	8.15
B	2.00	2.036	1.80	0.50	0.51	3.20
	2.00	1.998	-0.10	0.50	0.52	5.80
P	10.00	10.294	2.94	0.50	0.56	13.80
	10.00	10.098	0.98	0.50	0.63	26.00
Fe	10.00	10.271	2.71	0.50	0.55	9.90
	10.00	9.977	-0.23	0.50	0.54	9.40
Zn	10.00	10.309	3.09	0.50	0.76	52.80
	10.00	9.919	-0.81	0.50	0.69	39.80
Cu	10.00	10.216	2.16	0.50	0.51	3.60
	10.00	9.938	-0.62	0.50	0.52	4.80
Mn	10.00	10.358	3.58	0.50	0.51	2.80
	10.00	10.035	0.35	0.50	0.51	2.00
Al	2.00	2.056	2.80	0.02	0.02	25.00
	2.00	2.025	-	0.20	0.02	-90.00

A.3 Corn steep liquor medium (20 g/L)

The concentration profiles of other trace metals and elements as well as total nitrogen and carbon during the fermentation in 20 g/L CSL medium are shown below in figures A.5 and A.6. The concentration profiles of other trace metals and elements as well as total nitrogen and carbon during the fermentation in 20 g/L CSL medium are shown below.

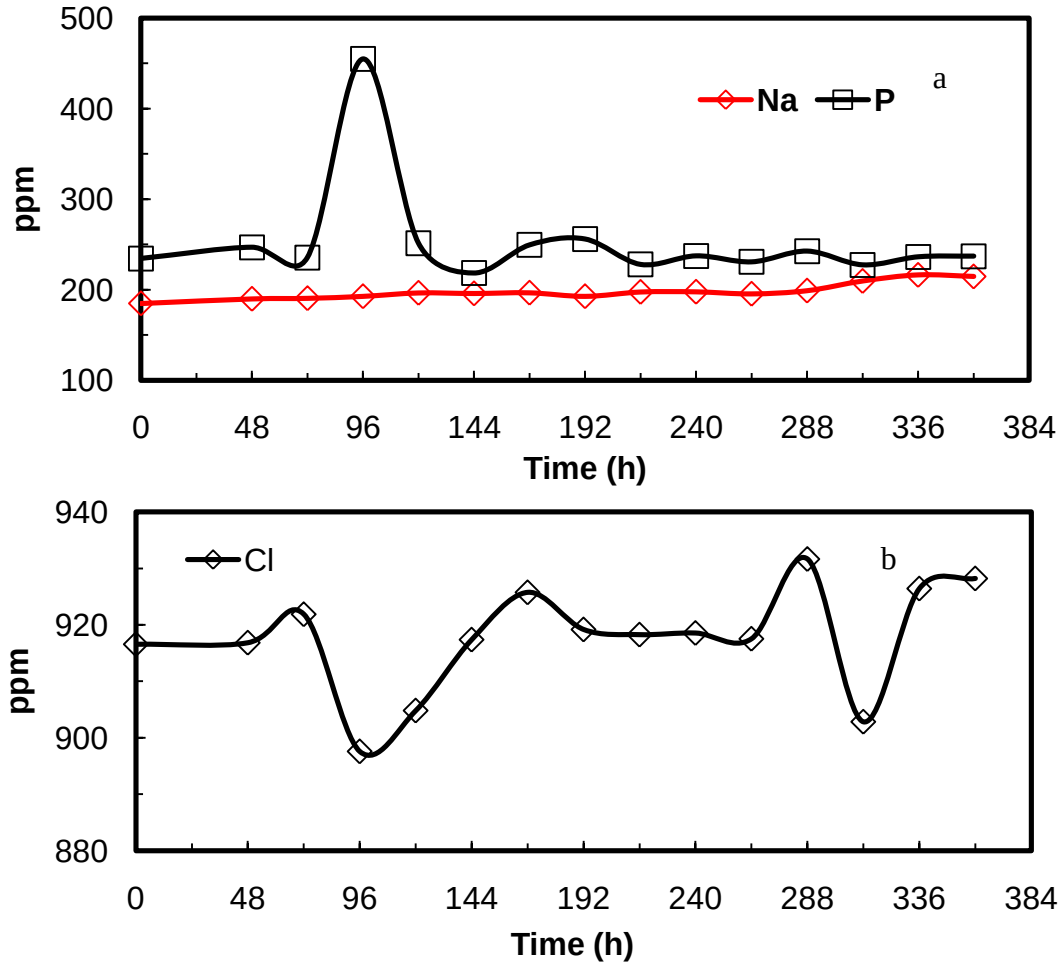

Figure A.5 Trace metals and minerals profiles during syngas fermentation by strain P11 in 20 g/L corn steep liquor medium.

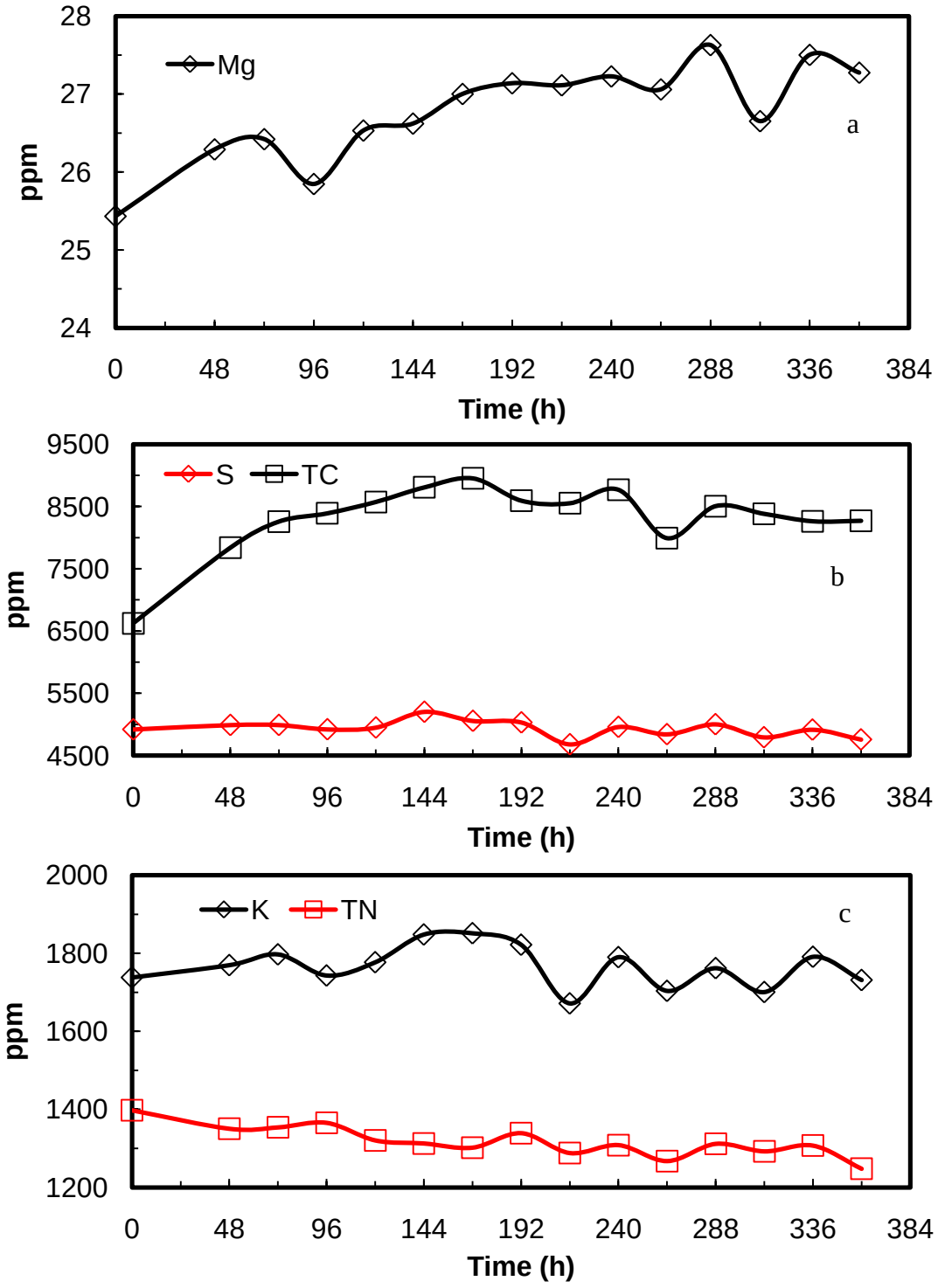

Figure A.6 Trace metals and minerals profiles during syngas fermentation by strain P11 in 20 g/L corn steep liquor medium.

Table A.3 Accuracy of analysis of trace metals and minerals on ICP for 20 g/L corn steep liquor medium for calibration standards and known samples.

Element	Calibration Standard (ppm)	True Value (ppm)	% error[a]	Known Concentration sample (ppm)	True Value (ppm)	% error[a]
As	0.45	0.43	-4.44	-	-	-
Ba	0.60	0.61	1.66	0.32	0.30	-3.81
Co	0.11	0.10	-9.09	0.37	0.35	-5.31
Cr	0.13	0.14	7.69	0.41	0.38	-5.23
Mo	0.43	0.43	0.00	0.40	0.21	-2.76
Ni	1.40	1.41	0.71	0.65	0.65	0.15
Se	0.54	0.51	-5.55	0.79	0.74	-5.32
Sr	0.21	0.21	0.00	0.17	0.16	0.00
Cl	902.80	901.00	-0.19	-	-	-
Na	100.00	99.21	-0.79	50.00	53.47	6.95
Ca	100.00	98.72	-1.28	50.00	52.59	5.18
Mg	100.00	98.09	-1.91	50.00	52.49	4.99
K	100.00	98.72	-1.28	50.00	52.71	5.52
S	100.00	97.27	-2.72	50.00	50.85	1.65
P	10.00	9.83	-1.71	0.50	0.59	11.80
Fe	10.00	9.79	-2.07	0.50	0.55	5.00
Zn	10.00	9.85	-1.49	0.50	0.59	18.60

a- percentage relative error

APPENDIX B

Amount of sugars consumed by strain P11 during syngas fermentation in 10 g/L and 20 g/L corn steep liquor (CSL) media

Fermentation samples from the 10 g/L and 20 g/L CSL media in 7.5 L fermentor were analyzed to calculate the sugars utilization. The sugar analysis was done using the HPLC as discussed in section 3.22. It can be seen that mainly glucose was consumed in 360 h (Tables B1 and B2). Data related to glucose was used for calculating ethanol from sugars in CSL. Only 10% and 76% of the total initial amounts of glucose in the 10 g/L CSL and 20 g/L CSL were consumed, respectively. The maximum possible ethanol production assuming all the glucose is consumed in the 10 g/L CSL and 20 g/L CSL media is below 0.16 g/L.

Table B.1 Amount of sugars consumed by strain P11 during syngas fermentation in 10 g/L corn steep liquor medium.

Time	Cellobiose (g/L)	Glucose (g/L)	Xylose (g/L)	Galactose (g/L)	Mannose (g/L)	Arabinose (g/L)	Total Sugars (g/L)
0	0.34	0.12	0.13	0.18	0.10	0.42	1.29
120	0.34	0.08	0.13	0.18	0.10	1.09	1.91
240	0.36	0.10	0.12	0.11	0.00	3.27	3.96
360	0.34	0.11	0.13	0.18	0.10	2.61	3.47

Table B.2 Amount of sugars consumed by strain P11 during syngas fermentation in 20 g/L corn steep liquor medium

Time	Cellobiose (g/L)	Glucose (g/L)	Xylose (g/L)	Galactose (g/L)	Mannose (g/L)	Arabinose (g/L)	Total Sugars (g/L)
0	0.66	0.32	0.16	0.13	0.07	0.05	1.38
120	0.67	0.26	0.16	0.13	0.00	1.14	2.35
240	0.67	0.19	0.16	0.13	0.00	3.25	4.0
360	0.61	0.08	0.12	0.19	0.00	2.56	3.56

APPENDIX C

Mathematical formulas used in the calculation of various fermentation parameters.

1. In batch fermentation mode, the rate of increase in cell concentration is proportional to cell concentration as shown in the equation below

$$\frac{dX}{dT} = \mu X \qquad (C.1)$$

Where,

dX/dT is rate if change in cell concentration

μ is cell specific growth rate (h^{-1})

X is cell mass concentration (g/L)

The specific growth rate is determined by integrating the above equation.

$$\mu = \frac{Ln\left(X/X_O\right)}{T - T_O} \qquad (C.2)$$

Where,

μ_{max} is maximum specific cell growth rate (h^{-1})

X is cell concentration at time T (g/L)

X_O is cell concentration at time T_O (g/L)

The maximum specific growth rate, μ_{max} is the slope of the straight line obtained by plotting ln (X/Xo) vs T using the data points in the exponential growth phase.

Sample calculation: The maximum specific growth rate of strain P11 during syngas fermentation in the 20 g/L CSL medium in the 7.5L fermentor is shown in Figure C.1. Slope of the line in the plot between ln (X/Xo) vs Time gives the maximum specific growth rate, which is 0.075 h^{-1}.

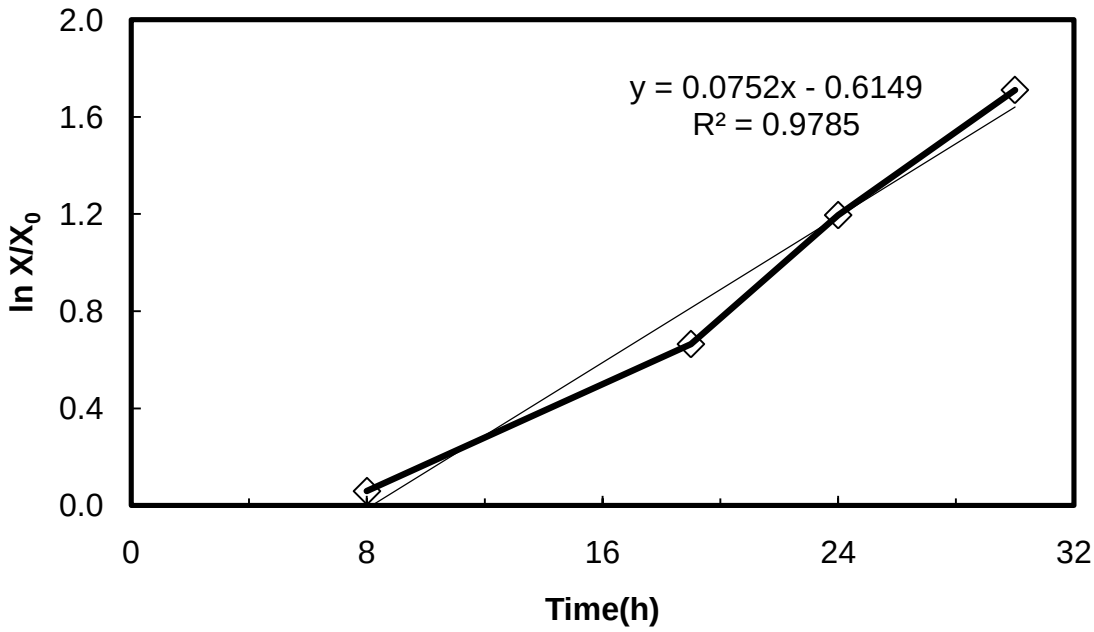

Figure C.1 Plot between ln (X/Xo) vs Time to calculate the specific growth rate of stain P11 in 20 g/L CSL medium.

2. Specific gas uptake rate (q_i)

$$q_i = \frac{n}{\frac{X}{V}*(T_{24(a+1)} - T_{24a})} \quad \text{(C.3)}$$

Where,

q_i is the specific gas (i) uptake rate (mole gas (i)/g cell/h)

n is the moles of gas (i) consumed between two time intervals

$T_{24(a+1)} - T_{24a}$ term is the time interval between each gas sampling where a = 0 to 24 h

X is cell concentration at time T (g/L)

V is volume of the fermentation broth at time T (L)

3. Percentage of CO utilization = $\frac{CO_{in} - CO_{out}}{CO_{in}} * 100$ (C.4)

$$N_{CO_{in}} = \frac{P * V * Y_{in}}{R * T}$$

$$\text{and } N_{CO_{out}} = \frac{P * V * Y_{out}}{RT}$$

Where,

NCO_{in} is the total moles of CO supplied to the fermentor (mol)

NCO_{out} is total moles of CO present in the exhaust stream (mol)

P is the pressure in the reactor (kPa)

V is the volumetric flow rate of gas in the reactor (sccm) or volume of the gas in the head space for experiments in serum bottles (mL)

y_i and y_{out} are the mole faction of CO in the feed and outlet streams.

T is the room temperature in K at which gas analysis is performed.

R is the universal gas constant (kPa L/ mol K).

Sample calculation of % CO utilization in 20 g/L CSL medium in 7.5 L fermentor:

Raw data: Co_{in} = 37.76 moles, CO_{out} = 31.97 moles

$$\% \text{ CO utilized} = \frac{(37.76 - 31.97)}{37.76} * 100 = 15.33\%$$

4. Product yield coefficient from CO ($Y_{P/S}$)

$$(Y_{P/S}) = \frac{\text{Total moles of product formed}}{\text{Total moles of CO consumed}} \quad \text{(C.5)}$$

Sample calculation: Ethanol yield by strain P11 during syngas fermentation in the 20 g/L CSL medium in the 7.5 L fermentor.

Raw data: Total moles of ethanol produced at time (360 h) = 0.58 moles

Total moles of CO consumed = 5.8 moles

$$(Y_{Ethanol/CO}) = \frac{0.58}{5.8} = 0.1$$

5. Percentage of ethanol yield = $\frac{\text{Actual yield } \left(Y_{P/S}\right)}{\text{Theoritical yield}} * 100\ \%$ (C.6)

Sample calculation: % ethanol yield from CO during syngas fermentation by strain P11 in the 20 g/L CSL medium in the 7.5 L fermentor.

Raw data: Actual yield = 0.1

Theoretical ethanol yield from CO (Equation 3.1) = 0.167

$$\%\ \text{yield of ethanol} = \frac{0.1}{0.167} * 100 = 60\%$$

6. Productivity of ethanol (g ethanol/L·h) = $\frac{\text{Amount of ethanol produced}}{\text{Working volume of reactor*Time}}$ (C.7)

Sample calculation: Productivity of ethanol by strain P11 during syngas fermentation in the 20 g/L CSL medium in the 7.5 L fermentor.

Raw data: Amount of ethanol in 3.3 L of fermentation medium at 360 h = 9.6 g/L

$$\text{Productivity of ethanol (g ethanol/L·h)} = \frac{9.6}{360} = 0.0267 \text{ g ethanol/L·h}$$

APPENDIX D

Model SAS program for determining least significant difference ($p < 0.05$)

PROGRAM: Below is the SAS program used for determining the significant difference in the amount of ethanol produced in 10 g/L and 20 g/L CSL media compared to 1 g/L yeast extract medium after 600 h of fermentation. (The statistical analyses presented in the Tables are for the fermentation results at 600 h).

```
options ls=74 ps=60;
data CSLeth25;
infile "H:\SAS\all data\ethanol25.csv" dlm=",";
input trt$ block rep e25;
cards;
run;
proc glm data=CSLeth25; class trt block;
model e25 = trt;
means trt/dunnett('YE 1.0');
run;
```

SAMPLE SAS OUTPUT

The GLM Procedure
Class Level Information

Class	Levels	Values
trt	3	CSL 10.0 CSL 20.0 YE 1.0
block	1	1

Number of Observations Read	9
Number of Observations Used	9

The GLM Procedure

Dependent Variable: e25

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	3.22604156	1.61302078	5.17	0.0496
Error	6	1.87286867	0.31214478		
Corrected Total	8	5.09891022			

R-Square	Coeff Var	Root MSE	e25 Mean
0.632692	30.07892	0.558699	1.857444

Source	DF	Type I SS	Mean Square	F Value	Pr > F
trt	2	3.22604156	1.61302078	5.17	0.0496

Source	DF	Type III SS	Mean Square	F Value	Pr > F
trt	2	3.22604156	1.61302078	5.17	0.0496

The GLM Procedure

Dunnett's t Tests for e25

NOTE: This test controls the Type I experiment wise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.312145
Critical Value of Dunnett's t	2.86275
Minimum Significant Difference	1.3059

Comparisons significant at the 0.05 level are indicated by ***.

trt Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
CSL 20.0 - YE 1.0	1.3623	0.0564	2.6683	***
CSL 10.0 - YE 1.0	0.2110	-1.0949	1.5169	

RESULTS:

The treatment with "YES" shown in the Tables were significantly different compared to the other medium ($p < 0.05$).

Table D.1: Summary of statistical analysis for ethanol concentrations and their significance between different media used at time = 600 h.

Treatment	Lower limit	Upper limit	Mean	Significantly different treatment
10 g/L CSL vs 1 g/L YE	-1.0949	1.5169	0.2110	NO
20 g/L CSL vs 1 g/L YE	0.0564	3.3196	1.3623	YES
20 g/L CSL vs 10 g/L CSL	-0.1546	2.4573	1.1513	NO

The amount of ethanol produced after 600h of fermentation in the 20 g/L CSL medium was significantly higher compared to the 1 g/L YE medium used ($P < 0.05$).

However, there was no significant difference in the amount of ethanol produced in the 10 g/L CSL medium compared to either the 20 g/L CSL or the 1 g/L YE medium.

Table D.2: Summary of statistical analysis for acetic acid concentrations and their significance between different media used at time = 600 h.

Treatment	Lower limit	Upper limit	Mean	Significantly different treatment
10 g/L CSL vs 1 g/L YE	-1.1954	0.7314	-0.2320	NO
20 g/L CSL vs 1 g/L YE	-2.6714	-0.7446	-1.7080	YES
20 g/L CSL vs 10 g/L CSL	-2.4394	-0.5126	-1.476	YES

The acetic acid concentrations in 1 g/L YE and 10 g/L CSL media were significantly higher compared to 20 g/L CSL medium at 600 h ($P < 0.05$). However, there was no significant difference in acetic acid concentrations between 1 g/L YE and 10 g/L CSL media.

Table D.3: Summary of statistical analysis for cell concentrations and their significance between different media used at time = 600 h.

Treatment	Lower limit	Upper limit	Mean	Significantly different treatment
10 g/L CSL vs 1 g/L YE	0.08962	0.36572	0.22767	YES
20 g/L CSL vs 1 g/L YE	0.01562	0.29172	0.15367	YES
20 g/L CSL vs 10 g/L CSL	-0.21205	0.06405	-0.07400	NO

The cell concentration at 600 h in 10 g/L and 20 g/L CSL media were significantly higher compared to the 1 g/L YE medium ($P < 0.05$). However, there was no significant difference in cell concentration between the CSL media used.

Table D.4: Summary of statistical analysis for butanol concentrations and their significance between different media used at time = 600 h.

Treatment	Lower limit	Upper limit	Mean	Significantly different treatment
10 g/L CSL vs 1 g/L YE	0.13587	0.69880	0.41733	YES
20 g/L CSL vs 1 g/L YE	0.28253	0.84547	0.56400	YES
20 g/L CSL vs 10 g/L CSL	-0.13480	0.42813	0.14667	NO

There was no significant difference in butanol concentration at 600 h between 10 g/L and 20 g/L CSL media ($P < 0.05$). However, the amount of butanol produced in 10 g/L and 20 g/L CSL media was significantly higher compared to the 1 g/L YE medium.

VITA

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Title of Study: ETHANOL PRODUCTION FROM SYNGAS BY *CLOSTRIDIUM* STRAIN P11 USING CORN STEEP LIQUOR AS A NUTRIENT REPLACEMENT

Pages in Study: 164 Candidate for the Degree of Master of Science

Major Field: Biosystems Engineering

Scope and Method of Study:

The objective of this research is to evaluate corn steep liquor (CSL) as an inexpensive nutrient and substitute to yeast extract (YE) used in the conversion of synthesis gas (syngas) to ethanol. Syngas is primarily a mixture of CO, CO_2, and H_2. CSL is rich in carbohydrates, vitamins, minerals, and trace metals and is much lower in cost compared to YE. Yeast extract was employed at a concentration of 1 g/L, while two CSL concentrations (10 g/L and 20 g/L) were investigated. Initially, growth and product profiles of *Clostridium* strain P11 in YE and CSL media were followed during fermentation in 250-mL serum bottles fed with syngas every 24 h at 239 kPa (absolute). In subsequent runs, the syngas fermentation was scaled-up to 7.5-L fermentor, in which growth, product profile and redox potential were closely monitored during the fermentation process.

Findings and Conclusions:

After 600 h of fermentation, ethanol concentrations in 250-mL serum bottles with 1 g/L YE, 10 g/L and 20 g/L CSL were 1.3 g/L, 1.5 g/L, and 2.7 g/L, respectively. Ethanol yields in these media after 336 h were 72%, 79% and 142% of the theoretical values based on CO consumed, respectively. The maximum ethanol concentrations after 360 h of fermentation in 7.5-L fermentor with 10 g/L and 20 g/L CSL media were 8.6 g/L and 9.6 g/L, respectively, which represent 57% and 60% of the theoretical ethanol yields. Only about 6.1 g/L of ethanol was obtained in the medium with 1 g/L YE after 360 h, which represents 53% of the theoretical ethanol yield.

These results showed that CSL can be used as a substitute for YE in syngas fermentation. In addition, CSL can enhance ethanol production and provide substantial savings by reducing the cost of the medium, which is an important factor in large scale ethanol production.

ADVISER'S APPROVAL: Dr. Hasan K. Atiyeh